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# Quantitative trait locus mapping for seed mineral concentrations in two *Arabidopsis thaliana* recombinant inbred populations

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## Summary

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- Biofortification of foods, achieved by increasing the concentrations of minerals such as iron (Fe) and zinc (Zn), is a goal of plant scientists. Understanding genes that influence seed mineral concentration in a model plant such as *Arabidopsis* could help in the development of nutritionally enhanced crop cultivars.
- Quantitative trait locus (QTL) mapping for seed concentrations of calcium (Ca), copper (Cu), Fe, potassium (K), magnesium (Mg), manganese (Mn), phosphorus (P), sulfur (S), and Zn was performed using two recombinant inbred line (RIL) populations, Columbia (Col) × Landsberg *erecta* (Ler) and Cape Verde Islands (Cvi) × Ler, grown on multiple occasions. QTL mapping was also performed using data from silique hulls and the ratio of seed:hull mineral concentration of the Cvi × Ler population.
- Over 100 QTLs that affected seed mineral concentration were identified. Twenty-nine seed QTLs were found in more than one experiment, and several QTLs were found for both seed and hull mineral traits. A number of candidate genes affecting seed mineral concentration are discussed.
- These results indicate that *A. thaliana* is a suitable and convenient model for discovery of genes that affect seed mineral concentration. Some strong QTLs had no obvious candidate genes, offering the possibility of identifying unknown genes that affect mineral uptake and translocation to seeds.

**Key words:** biofortification, copper (Cu), *erecta*, iron (Fe), mineral partitioning, seed mineral concentration, zinc (Zn).

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## Introduction

On a worldwide basis, plants are an important source of human food. Plant-based foods often have low mineral density and, as a result, a large proportion of the world's population suffers from mineral malnutrition, especially for iron (Fe) and zinc (Zn). In recent years, plant scientists have adopted a strategy known as biofortification in order to address this problem (Grusak & DellaPenna, 1999; White &

Broadley, 2005). The goal of biofortification is to increase nutrient density in the edible portions of crop plants, which for many important staple crops, such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), maize (*Zea mays*), bean (*Phaseolus vulgaris*) and other legumes, are seeds.

Achieving biofortification of crops is a major challenge. The physiology and regulation of mineral uptake and translocation to seeds in plants are not well understood (Briat *et al.*, 2007; Krämer *et al.*, 2007; Zhang *et al.*, 2007). Thus, it is unclear which genes should be targeted in breeding programs or in transgenic efforts to accomplish crop improvement. Additionally, it is unknown what other modifications may be needed directly in seeds to allow accumulation of those

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minerals that are potentially toxic to the plant at elevated concentrations. Use of a small, fast-growing model plant, such as *Arabidopsis thaliana*, for discovery of genes that affect seed mineral concentration could save considerable time and effort as compared to working directly in crops that require more time, space, and labor to grow. *Arabidopsis thaliana* is closely related to the *Brassica* genus, which includes several important crop species. Seeds of some of these crops, such as broccoli (*Brassica oleracea*), are consumed as sprouts, and rapeseed (*Brassica napus*) meal is commonly used in animal feed or as an oil source. Additionally, genetic and genomic resources for *A. thaliana* are highly developed and available to the plant science community.

Initial biofortification efforts have focused on overexpression of single genes to increase mineral uptake or storage (Goto *et al.*, 1999; Vasconcelos *et al.*, 2003; Ramesh *et al.*, 2004; Vasconcelos *et al.*, 2004, 2006). Analysis of mineral overaccumulation mutants indicates that translocation of minerals to seeds is tightly regulated, and that simply increasing uptake into the plant will probably not result in seeds with higher mineral contents or concentrations. It is likely that multiple genes will need to be overexpressed in tandem or at appropriate developmental stages in order to increase both mineral intake into the plant and mineral translocation to the target tissues. These targets may include not only genes that increase mineral uptake, but also genes for internal transporters, such as for vascular tissue loading or unloading, or for organelle influx or efflux. Another category of potential target genes includes metal chaperones or chelators that are necessary for metal transport, storage, or detoxification. Regulatory genes that alter expression of entire pathways are another potential category of target genes.

Molecular genetics or genomic approaches have been used in preliminary steps to identify genes that allow certain plants to highly accumulate minerals such as cesium (Payne *et al.*, 2004), selenium (Zhang *et al.*, 2006) and Zn (Filatov *et al.*, 2007). Microarray comparisons of hyperaccumulators and nonhyperaccumulators have revealed many genes that are differentially expressed in these plants (Becher *et al.*, 2004; Weber *et al.*, 2004; Filatov *et al.*, 2006; Hammond *et al.*, 2006; Talke *et al.*, 2006; van de Mortel *et al.*, 2006). Quantitative trait locus (QTL) mapping has been performed in bean (Guzmán-Maldonado *et al.*, 2003; Gelin *et al.*, 2007) and rice (Stangoulis *et al.*, 2007), and in an *A. thaliana* recombinant inbred line (RIL) population to identify loci that influence seed phosphorus (P) (Bentsink *et al.*, 2003) and other mineral characteristics (Vreugdenhil *et al.*, 2004). In this work, we extend the use of the model plant *A. thaliana* as a source of discovery of genes that alter seed mineral concentrations. We present QTL mapping data for two growth cycles of the Cape Verde Islands (Cvi) × Landsberg *erecta* (Ler) population and three cycles of the Columbia (Col) × Ler population to examine the reproducibility of QTL results. Additionally, QTLs were mapped for mineral concentrations in silique hulls and the ratios of mineral concentrations in mature seeds:hulls.

## Materials and methods

### Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. were obtained from the Arabidopsis Biological Resource Center at The Ohio State University, USA. In addition to RIL populations Col × Ler (CS1899) and Cvi × Ler (CS22000), *erecta* mutant lines *er*-114 (CS3918), *er*-116 (CS3920), *er*-117 (CS3921), and *er*-123 (CS3927), and T-DNA lines *heavy metal P1b-ATPase 5* (*hma5*; SALK\_040252C), *yellow stripe-like 8* (*ysl8*; CS859713), *ferritin 2* (*fer2*; SALK\_002947), and *zinc regulated transporter, iron regulated transporter-like protein 5* (*zip5*; SALK\_009007) were grown. Seeds were placed in 0.1% agar at 4°C for 3–5 d before sowing on commercial potting mix (MetroMix 300; Sun Gro Horticulture, Bellevue, WA, USA). For all QTL studies and experiments with T-DNA lines, plants were grown in an air-conditioned glasshouse under shaded cloth (to reduce sunlight intensity) during winter months for the 2005 and 2006 experiments, and during late winter/early spring months for the 2003 and 2007 experiments. Fluorescent lighting was supplied at approx. 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for a 16-h photoperiod. Water and nutrients were provided by subirrigation as needed (usually twice per week) as a solution of the following composition: 1.2 mM  $\text{KNO}_3$ , 0.8 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.8 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.3 mM  $\text{KH}_2\text{PO}_4$ , 0.2 mM  $\text{MgSO}_4$ , 25  $\mu\text{M}$   $\text{CaCl}_2$ , 25  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 2  $\mu\text{M}$   $\text{MnSO}_4$ , 2  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.5  $\mu\text{M}$   $\text{CuSO}_4$ , 0.5  $\mu\text{M}$   $\text{H}_2\text{MoO}_4$ , 0.1  $\mu\text{M}$   $\text{NiSO}_4$  and 10  $\mu\text{M}$  Fe-EDDHA as Sprint 138 (Becker-Underwood, Ames, IA, USA). For low-nutrient treatments in *erecta* mutant experiments, plants received nutrient solution once, at 21 d after sowing, and deionized water at all other times. For high-light treatments in *erecta* mutant experiments, plants were removed from the shaded cloth area after 2 wk and grown under ambient sunlight with supplemental lighting supplied by metal halide lamps on a 15-h photoperiod. Plants of each RIL were sown in three pots at a density of 3–5 plants per pot. At maturity, seeds from all plants were bulked, and a minimum of two replicate subsamples were used for inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis. Silique valves or hulls (when collected) were collected at the same time as seeds collected for mineral analysis. Silique hulls were carefully cleaned and inspected to remove seed, floral, and leaf tissue.

### Mineral analysis

Plant tissues were oven-dried at 60°C for 48 h before determination of dry weight (DW). Samples of 0.1–0.25 g were digested in nitric-perchloric acid (4 : 1) using a ramped heating protocol going from 100 to 220°C, and remaining at 220°C until samples were taken to dryness. Residues were re-suspended in 15 ml of 2% nitric acid. All acids were trace metal grade (Fisher Scientific, Pittsburgh, PA, USA) and water was filtered through a MilliQ system (Millipore, Billerica,

**Table 1** Mineral concentrations of seeds and hulls of *Arabidopsis thaliana* Columbia (Col) × *Ler* (CL) and *Cvi* × *Ler* (CVL) recombinant inbred line (RIL) populations

Mineral ( $\mu\text{g g}^{-1}$ )	Experiment					
	CVL 2003	CVL 2006	CVL 2006 hulls	CL 2003	CL 2005	CL 2007
Ca	2630–8296	2475–6061	21 009–40 619	3455–7872	2882–6418	3489–6634
Cu	3.98–13.39	3.69–13.5	2.2–9.4	3.4–10.7	2.9–8.6	2.9–8.4
Fe	57.2–158.0	50.7–144.7	14.9–122.6	55.1–117.7	48.0–148.1	66.2–151.9
K	6122–20 791	7841–18 455	24 629–82 339	7662–17 450	6482–16 409	6917–14 034
Mg	2596–4143	2559–4208	3225–12 137	2855–4029	2805–4046	3242–4773
Mn	19.2–40.9	11.9–28.2	3.5–14.9	17.3–34.3	23.9–65.7	13.9–38.9
P	6253–12 444	5955–12 280	2430–13 039	6914–11 262	6606–10 370	6738–11 616
S	8048–14 360	6824–12 575	2614–13 172	9230–14 835	4378–12 939	6854–11 846
Zn	30.2–86.1	46.5–111.9	22.4–81.5	33.3–64.1	28.4–80.3	38.0–71.7

Data are for seeds unless otherwise stated.

MA, USA) to at least 18 M $\Omega$  resistivity. Concentrations of calcium (Ca), copper (Cu), Fe, potassium (K), magnesium (Mg), manganese (Mn), P, sulfur (S), and Zn were determined by ICP-OES (CIROS ICP Model FCE12; Spectro, Kleve, Germany).

### QTL mapping and candidate gene selection

The Col × *Ler* (Lister & Dean, 1993) and *Cvi* × *Ler* (Alonso-Blanco *et al.*, 1998) RIL populations were previously mapped. For seed mineral concentrations, in 2003 and 2007, 100 lines of the Col × *Ler* population were analyzed, and in 2005, 97 lines were analyzed. In 2003, 159 lines of the *Cvi* × *Ler* population were analyzed for seed mineral concentrations, and in 2006 146 lines were analyzed for seed mineral concentrations and 84 lines were analyzed for silique hull mineral concentrations. Genetic markers and comparisons of these genetic maps are available on the Natural-EU project website (<http://www.dpw.wau.nl/natural/>). QTLs were mapped by composite interval mapping using WINQTL CARTOGRAPHER (Wang *et al.*, 2007). A likelihood ratio (LR) significance threshold of  $P = 0.05$  was determined for each trait by performing 1000 permutations before mapping (Supporting Information Table S1). Genetic markers that have been anchored to the physical map for *Cvi* × *Ler* (Peters *et al.*, 2001) and Col × *Ler* ([www.arabidopsis.org](http://www.arabidopsis.org)) were used to estimate the boundaries of confidence intervals. Annotated genes known to be involved with mineral uptake or mineral homeostasis (or family members of such genes) that fell within these confidence intervals were considered to be candidate genes.

### Results

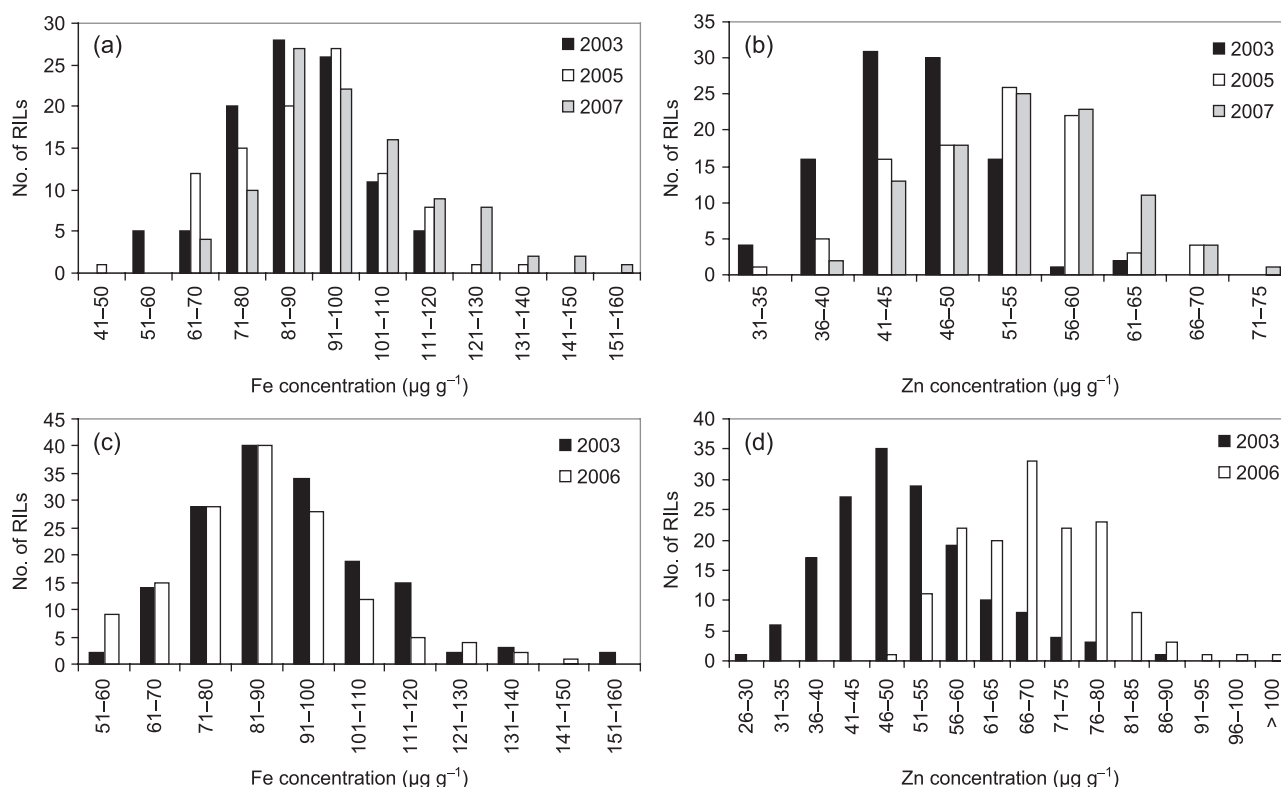
The Col × *Ler* RIL (CL) population was grown on three occasions (2003, 2005 and 2007), and the *Cvi* × *Ler* RIL (CVL) population was grown on two occasions (2003 and 2006). Seed mineral concentrations of both populations

and all experiments exhibited wide ranges from low to high (Table 1). The ranges were lowest for Mg and highest for Cu, and were generally consistent between occasions. Silique hulls were collected and analyzed for CVL in 2006. Silique hulls had wider mineral concentration ranges than did seeds, except for Ca. Hull values were much higher than seed values for Ca, K, and Mg, as expected (Waters & Grusak, 2008).

Although mineral trait ranges for populations were sometimes shifted upward in some experiments, the distribution of trait values among individual lines fell into the expected normal distribution pattern. For example, the frequency distribution of Fe concentration in both RIL populations was quite similar between experiments (Fig. 1a,c), while most lines had a lower Zn concentration in 2003 in both populations than in other experiments (Fig. 1b,d). Frequency distributions for the remaining seed minerals are shown in Supporting Information Figs S1 and S2. Silique hull mineral concentrations also had normal frequency distributions, although a few lines had substantially higher Cu and Fe concentrations than the majority of lines (Supporting Information Fig. S3). Correlations of seed mineral concentrations of individual RILs from each growth cycle are presented in Supporting Information Figs S4–S6.

Within each population and experiment, some seed mineral concentrations were consistently highly correlated, for example, the minerals Fe and Zn (Fig. 2). Cu and Zn were also consistently highly correlated, as were Mg and P (Supporting Information Table S2), but most minerals had weak positive correlations with other minerals, with a few exhibiting weak negative correlations. Correlations of mineral concentrations in silique hulls were weaker, with Mn and Zn exhibiting the strongest correlation.

QTL mapping results for the CL population are presented in Table 2 and Supporting Information Fig. S7. Several QTLs were mapped in all three experiments. These include QTLs on chromosome 2 for Ca, Cu, and P, and a QTL for seed S on chromosome 4. All of these QTLs had additive effects large enough, explaining at least 15% of the total variation, to



**Fig. 1** Histograms of seed iron (Fe) and zinc (Zn) concentrations in *Arabidopsis thaliana* Columbia (Col)  $\times$  Landsberg *erecta* (Ler) (CL) and Cape Verde Islands (Cvi)  $\times$  Ler (CVL) recombinant inbred line (RIL) populations. (a) Frequency distribution of seed Fe concentration in the CL population. (b) Frequency distribution of seed Zn concentration in the CL population. (c) Frequency distribution of seed Fe concentration in the CVL population. (d) Frequency distribution of seed Zn concentration in the CVL population.

potentially allow fine mapping of the quantitative gene in the QTL region. Several other QTLs for Ca, Cu, K, Mg, and Mn were mapped in two of the three experiments. The majority of QTLs mapped were found in only one experiment. For the seed traits of the CVL population (Table 3 and Supporting Information Fig. S8), we compared the results of our two experiments with each other and with a previous publication on this population (Vreugdenhil *et al.*, 2004). Several QTLs were mapped in all three experiments, including QTLs for Zn on chromosomes 1 and 2, one for K on chromosome 2, one for Mn on chromosome 1, and a P QTL on chromosome 3. The P QTL was quite strong, explaining 43 and 54% of trait variability in our two experiments. Twelve QTLs were mapped in two out of the three experiments; on chromosome 1, QTLs for Cu, S, and P; on chromosome 2, QTLs for Mg and P; on chromosome 3, QTLs for Mg, Mn, and S; and on chromosome 5, QTLs for Ca, S, and Zn. It should be noted that seed S was not studied by Vreugdenhil *et al.* (2004).

We also mapped QTLs for silique hull mineral concentration and seed:hull concentration ratio (Table 4 and Supporting Information Fig. S9) for CVL in 2006. One QTL, for P on chromosome 3, was mapped in all experiments (seeds, hulls, and seed:hull concentration ratio), while another QTL for Zn on chromosome 2 was mapped in all three seed mineral experi-

ments and in silique hulls. QTLs that were found in both our experiments and mapped previously include Mn and Zn on chromosome 1, K on chromosome 2, and Ca on chromosome 3. Several other QTLs were mapped for both seed and hull traits.

By anchoring the genetic markers used for map construction with known positions on the physical map, we were able to estimate which known or predicted genes fall within the QTL confidence intervals. Genes with known or predicted functions that could influence seed mineral traits were designated as candidate genes (Table 5). Four single-gene mutants with T-DNA insertions in candidate genes were analyzed for seed mineral concentrations. There were no significant differences in *hma5*, *ysl8*, or *zip5*, but *fer2* had approx. 10% lower seed Fe concentration.

Several of the QTLs mapped to chromosome 2 coincided with the *Erecta* (*ER*) locus (50.6 cM in CL; 49 cM in CVL). To test whether the *ER* locus was a quantitative trait gene, we grew four *er* mutant lines (*er-114*, *er-116*, and *er-117* in the Col-0 background, and *er-123* in the Wassilewskija-2 (Ws-2) background) and the appropriate wild-type lines under different conditions and quantified seed mineral concentrations (Table 6). Under high-light, high-nutrient conditions, Ca, Cu, Mg, P, and S were unchanged or significantly lower in the *er* mutants in the Col background, while Cu, Mg, P, and Zn

**Table 2** Significant quantitative trait loci (QTLs) for seed mineral concentration traits in the *Arabidopsis thaliana* Columbia (Col) × Ler (CL) recombinant inbred line (RIL) population

QTL no.	Chrom.	Trait	2003			2005			2007		
			CI (peak) <sup>a</sup>	% expl. <sup>b</sup>	Add. <sup>c</sup>	CI (peak) <sup>a</sup>	% expl. <sup>b</sup>	Add. <sup>c</sup>	CI (peak) <sup>a</sup>	% expl. <sup>b</sup>	Add. <sup>c</sup>
CL1	2	Ca							0–13 (11)	9	–
CL2	2	Ca	40–52 (50.6)	17	–	46.5–56.8 (50.6)	30	–	44.5–56.8 (50.6)	25	–
CL3	2	Ca	60	9	–	60	12	–	60	10	–
CL4	4	Ca	44.5–64.2 (54.6)	21	+				44.5–64.2 (54.6)	25	+
CL5	4	Ca				63.7–74.2 (67.9)	9	+			
CL6	5	Ca	141	9	+						
CL7	2	Cu	38.2–54.6 (48.8)	24	–	44.4–60 (50.6)	22	–	40.4–60 (50.6)	27	–
CL8	2	Cu				69.9– <u>71.4</u>	12	+			
CL9	3	Cu				8.4– <u>11</u>	9	+			
CL10	3	Cu				15.7– <u>18.6</u>	8	+			
CL11	3	Cu							55.3–66.6 (55.9)	9	+
CL12	5	Cu				90.6–111.7 (104.6)	26	–	100.6–120.4 (111.7)	15	–
CL13	1	Fe				0–1	10	–			
CL14	3	Fe				8.4–22.6 (11)	18	+			
CL15	4	Fe	0–8.3 (5.1)	14	–						
CL16	5	Fe							25.7–35.9 (29.6)	11	–
CL17	5	Fe	67.2–70.4 (68.4)	9	+						
CL18	5	Fe				90– <u>91</u>	10	+			
CL19	5	Fe				95.1–107.4 (98.9)	14	–			
CL20	1	K				22.6	8	–			
CL21	2	K				33.1– <u>35.1</u>	9	+			
CL22	2	K				38.2–44.5 (42.4)	11	+			
CL23	2	K	40.4–60 (50.6)	53	–						
CL24	2	K	65.2–86.5 (69.9, 75.8, 84.5)	25	+	65.2–73.8 (71.4)	10	+			
				27	+						
				18	+				76.1–94 (86.1)	24	+
CL25	5	K	80.8–88.2 (84.4)	10	–						
CL26	1	Mg				36.6–43.1 (39.6)	17	–			
CL27	1	Mg				69.3–72.9 (70.9)	10	+			
CL28	2	Mg	40.4–60 (50.6)	22	–						
CL29	3	Mg							10.4–17.7 (15.5)	12	+
CL30	3	Mg	26.6–41.2 (36.3)	10	+				<u>20.6</u> –30	13	+
CL31	5	Mg	117.5–134.4 (127.1)	11	–						
CL32	1	Mn	<u>16.7</u> –17	10	–						
CL33	1	Mn	55.7	10	+						
CL34	2	Mn				40.4–58 (50.6)	15	–	40.4–60 (50.6)	17	–
CL35	1	P	69.3–78.7 (70.9)	8	+						
CL36	1	P				78.7–91.2 (87.2)	11	+			
CL37	1	P							<u>118.3</u> –120.3	11	+
CL38	2	P	40.4–60 (50.6)	37	–	46.5–56.8 (50.6)	19	–	48.8–52.6 (50.6)	15	–
CL39	1	S				0–4 (1.9)	11	–			
CL40	2	S	46.5–52.6 (50.6)	10	–						
CL41	3	S							20.6–30 (22.6)	9	–
CL42	3	S	<u>34.9</u> –36.3	7	–						
CL43	3	S	40– <u>41.2</u>	10	–						
CL44	4	S	3.1–23.4 (15.5)	25	+	8.3–30.1 (17.5)	22	+	13.5–30.1 (15.5)	31	+
CL45	5	S	14.3–23.7 (16.5)	9	+						
CL46	1	Zn	69.3– <u>70.9</u>	9	+						
CL47	2	Zn	44.5–52.6 (50.6)	16	–				44.5–60 (50.6)	33	–
CL48	5	Zn							25.7–40.8 (35.9)	11	–
CL49	5	Zn							44.2–46.6 (44.6)	7	–
CL50	5	Zn							100.6	6	–

<sup>a</sup>Confidence interval (CI) of QTL ( $P < 0.05$ ) in cM. The position of the peak logarithm of odds (LOD) score is in parentheses or underlined. Some QTLs contain multiple peaks.

<sup>b</sup>Percentage of variability explained by this trait ( $r^2$  at peak position).

<sup>c</sup>Additive effect. A negative value indicates that the Col allele decreases the trait value. Chrom., chromosome.

**Table 3** Significant quantitative trait loci (QTLs) for seed mineral concentration traits in the *Arabidopsis thaliana* Cvi × Ler (CVL) recombinant inbred line (RIL) population

QTL no.	Chrom.	Trait	2003			2006			Vr. <sup>d</sup>
			CI (peak) <sup>a</sup>	% expl. <sup>b</sup>	Add. <sup>c</sup>	CI (peak) <sup>a</sup>	% expl. <sup>b</sup>	Add. <sup>c</sup>	
CVL1	1	Ca				16–21 (18)	10	–	
CVL2	1	Ca	45–50 (47)	6	+				
CVL3	2	Ca	42–45 (43)	7	+				
CVL4	2	Ca				61–69 (67)	12	+	
CVL5	3	Ca	0–17 (9)	19	–	3–9 (8)	6	–	Yes
CVL6	5	Ca	0–10 (2)	10	+	0	7	+	
CVL7	1	Cu	83–103 (96)	16	–	90– <u>96</u>	8	–	
CVL8	2	Cu	37–58 (49)	24	+				
CVL9	2	Cu				67– <u>69</u>	8	–	
CVL10	4	Cu				47–55 (53)	7	–	
CVL11	4	Cu				63	8	–	
CVL12	5	Cu				37– <u>40</u>	7	+	
CVL13	1	Fe	98–106 (103)	6	–				
CVL14	2	Fe	35–58 (49)	18	+				
CVL15	3	Fe	9–29 (19)	16	–				
CVL16	5	Fe				35–46 (40)	6	+	
CVL17	1	K	75–82 (79)	5	–				
CVL18	2	K	45–58	15	+				
CVL19	2	K	65– <u>69</u>	19	–	65– <u>69</u>	10	–	Yes
CVL20	5	K				0–12	10	–	
CVL21	1	Mg	16–25 (23)	6	–				
CVL22	2	Mg	35–58 (51)	27	+	42– <u>51</u>	17	+	
CVL23	3	Mg	0–5	5	–	0–11	14	–	
CVL24	5	Mg	30–45 (32, 40)	6	+				
				8	+				
CVL25	5	Mg				68–71 (69)	5	+	
CVL26	1	Mn	104–124 (115)	17	–	122– <u>124</u>	9	–	Yes
CVL27	3	Mn	0–11 (3)	11	–				Yes
CVL28	5	Mn	5–6	5	+				
CVL29	5	Mn	90–107 (103)	11	+				
CVL30	1	P	0–11 (4)	4	–	4–8 (6)	4	–	
CVL31	1	P	16– <u>20</u>	4	–				
CVL32	1	P	51	2	–				
CVL33	2	P	35–58 (49)	13	+	37–58 (49)	7	+	
CVL34	3	P	0–11	43	–	0–11 (3)	54	–	Yes
CVL35	3	P	17	2	–				
CVL36	4	P	46–50 (48)	3	–				
CVL37	1	S	14–32 (23)	7	+	8–20 (11)	10	+	
CVL38	1	S	110–122 (115)	12.5	–				
CVL39	3	S	0–11	6	–	0–11	9	–	
CVL40	3	S	<u>72</u> –79	6	+				
CVL41	4	S				40–53 (47)	8	–	
CVL42	5	S	77–96 (89)	12	–	<u>87</u> –89	8	–	
CVL43	5	S				92–107 (99)	12	–	
CVL44	1	Zn	0–20 (8)	13	–	0–8 (4)	12	–	Yes
CVL45	2	Zn	35–58 (42, 49)	10	+	39–42 (40)	5	+	Yes
				15	+	47–54 (49)	7	+	
CVL46	3	Zn	0–11	10	–				
CVL47	5	Zn	20–39 (30, 37)	11	+				
				10	+				
CVL48	5	Zn				45–48 (46)	5	+	Yes
CVL49	5	Zn				80–105 (92)	14	+	

<sup>a</sup>Confidence interval (CI) of QTL ( $P < 0.05$ ) in cM. The position of the peak logarithm of odds (LOD) score is in parentheses or underlined. Some QTLs contain multiple peaks.

<sup>b</sup>Percentage of variability explained by this trait ( $r^2$  at peak position).

<sup>c</sup>Additive effect. A negative value indicates that the Col allele decreases the trait value.

<sup>d</sup>'Yes' indicates that a similar QTL was identified by Vreugdenhil *et al.* (2004) ('Vr.?').

Chrom., chromosome.

**Table 4** Significant quantitative trait loci (QTLs) for silique hull mineral concentrations and seed:hull mineral concentration ratio traits in the *Arabidopsis thaliana* Cvi × Ler (CVL) recombinant inbred line (RIL) population grown in 2006

QTL no.	Chrom.	Trait	Hulls			Seed:hull			Seed QTL? <sup>d</sup>
			CI (peak) <sup>a</sup>	% expl. <sup>b</sup>	Add. <sup>c</sup>	CI (peak) <sup>a</sup>	% expl. <sup>b</sup>	Add. <sup>c</sup>	
CVL50	1	Cu	92–98 (93)	8	–				2003, 2006
CVL51	2	Cu	39–58 (49)	28	+				2003
CVL52	2	Cu	67– <u>69</u>	10	–				2006
CVL53	3	Cu	<u>70</u> –78	7	–				
CVL54	5	Cu	32– <u>34</u>	12	+				2006
CVL55	2	K	32–51 (47)	12	+	34–54 (40)	20	–	2003
CVL56	5	K	<u>64</u> –66	14	–	64	9	+	
CVL57	5	Mg				30– <u>32</u>	13	+	2003
CVL58	2	P				67– <u>69</u>	12	+	
CVL59	3	P	0–11 (2)	43	–	<u>0</u> –11	18	+	2003, 2006, Vr.
CVL60	1	S				32–47 (38)	19	+	
CVL61	3	S	0	9	–				2003, 2006
CVL62	3	S	70	14	–				2003
CVL63	2	Zn	32–43 (34)	16	+				2003, 2006, Vr.
CVL64	5	Zn	55–61 (59)	16	+				

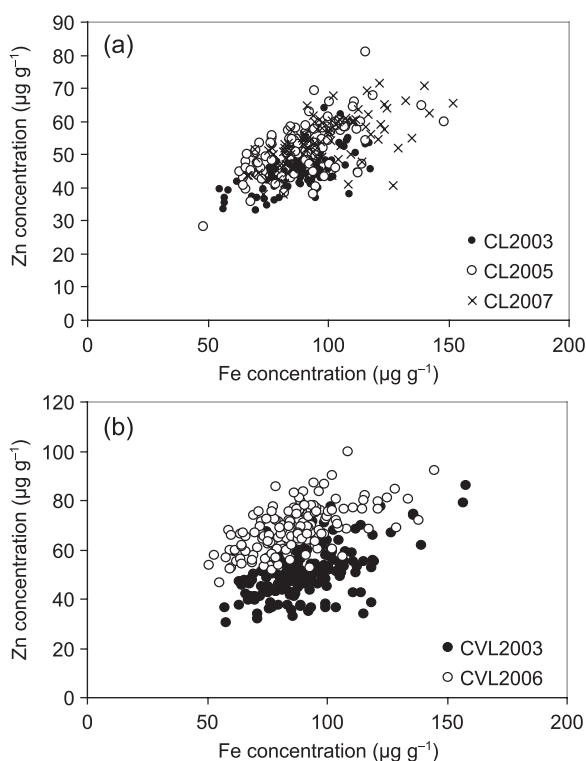
<sup>a</sup>Confidence interval (CI) of QTL ( $P < 0.05$ ) in cM. The position of the peak logarithm of odds (LOD) score is in parentheses or underlined. Some QTLs contain multiple peaks.

<sup>b</sup>Percentage of variability explained by this trait ( $r^2$  at peak position).

<sup>c</sup>Additive effect. A negative value indicates that the Col allele decreases the trait value.

<sup>d</sup>Overlapping QTLs identified in Table 3.

Chrom., chromosome; Vr., Vreugdenhil et al. (2004).

**Fig. 2** Correlations of seed iron (Fe) and zinc (Zn) concentrations in *Arabidopsis thaliana* Columbia (Col) × Ler (CL) and Cvi × Ler (CVL) recombinant inbred line (RIL) populations. (a) CL population correlations. (b) CVL population correlations.

were significantly higher in *er*-123. K was higher in two of the *er* lines, while Mn was lower in three of the four *er* lines. By contrast, when grown in low-light conditions (as the RIL populations were grown), with either low or high nutrients, in the majority of lines and plant growth cycles, Ca, Cu, K, and Zn were significantly higher in the *er* mutant lines relative to their wild-type parent. Seed Fe concentration was higher in six instances and lower in one, while Mg was higher in five of 12 instances. Seed Mn, P, and S were not consistently different from wild-type parent lines.

## Discussion

QTL mapping is a powerful genetic technique that can be used to identify markers or genes associated with a quantitative trait, such as seed mineral concentration. A gene within QTL confidence intervals is unlikely to be the sole determinant of the trait, as there are often multiple genes that affect the trait of interest through individual additive effects or interactions (Flint & Mott, 2001; Tonsor *et al.*, 2005). Movement of minerals from the soil into and through the plant to seeds requires an unknown number of membrane transport processes through multiple tissue types. As such, QTLs for increased seed mineral concentration may indicate genes that are important at any limiting step, such as uptake at the root surface, translocation (xylem loading/unloading or phloem loading/unloading), storage capacity (in source tissue or seed), and remobilization processes, or genes



**Table 5** Candidate genes for quantitative trait loci (QTLs), categorized by predicted function

Transporters				Chelators/storage			
QTL no.	Mineral trait	Gene	Locus ID	QTL no.	Mineral trait	Gene	Locus ID
CL23, CVL18, CVL55	K	<i>AKT1</i>	At2g26650	CVL7, CVL50	Cu	<i>ATX1</i>	At1g66240
CVL6	Ca	<i>CAX4</i>	At5g01490	CVL53	Cu	<i>CCH</i>	At3g56240
CL12	Cu	<i>COPT1</i>	At5g59030	CL8, CVL9, CVL52	Cu	<i>CCH-like</i>	At2g37390
CL7, CVL8, CVL51	Cu	<i>COPT1</i> family	At2g26975	CL12	Cu	<i>CCH-like</i>	At5g63530
CL11	Cu	<i>COPT2</i>	At3g46900	CL12	Cu	<i>CCH-like</i>	At5g66110
CL12	Cu	<i>COPT3</i>	At5g59040	CL14	Fe	<i>FER2</i>	At3g11050
CL8, CVL9, CVL52	Cu	<i>COPT4</i>	At2g37925	CL7, CVL8, CVL51	Cu	Cu-binding family	At2g28660
CVL12, CVL54	Cu	<i>COPT5</i>	At5g20650	CVL44	Zn	<i>MT1a</i>	At1g07600
CL17, CVL16	Fe	Ferroportin2	At5g26820	CVL44	Zn	<i>MT1c</i>	At1g07610
CL14	Fe	<i>FRD3</i>	At3g08040	<b>Regulators</b>			
CVL45, CVL63	Zn	<i>HMA4</i>	At2g19110	<b>QTL no.</b>	<b>Mineral trait</b>	<b>Gene</b>	<b>Locus ID</b>
CVL7, CVL50	Cu	<i>HMA5</i>	At1g63440	CVL39, CVL61	S	APS kinase	At3g03900
CL12	Cu	<i>HMA7/RAN1</i>	At5g44790	CL3	Ca	<i>CXIP4</i>	At2g28910
CVL12, CVL54	Cu	<i>HMA8/PAA2</i>	At5g21930	CL1	Ca	<i>CXIP4</i> -like	At2g01100
CL22, CVL18, CVL55	K	<i>KT6</i>	At2g25600	CVL33	P	<i>PHO2</i>	At2g33770
CL23, CVL18, CVL55	K	<i>KUP1</i>	At2g30070	CVL46	Zn	Zn-binding TF	At3g07780
CL24	K	<i>KUP2</i>	At2g40540	<b>Metabolism</b>			
CVL20	K	<i>KUP7</i>	At5g09400	<b>QTL no.</b>	<b>Mineral trait</b>	<b>Gene</b>	<b>Locus ID</b>
CL23, CVL19	K	<i>KUP11</i>	At2g35060	CL42	S	<i>APR1</i>	At4g04610
CVL24, CVL57	Mg	<i>MGT1</i> -like	At5g22830	CL44	S	<i>APS1</i>	At3g22890
CL30	Mg	<i>MRS2</i> family	At3g19640	CL7, CVL8, CVL51	Cu	<i>CSD2</i>	At2g28190
CL31	Mg	<i>MRS2</i> family	At5g64560	CL49, CVL47	Zn	<i>CSD3</i>	At5g18100
CL47	Zn	<i>MTPb1</i>	At2g29410	CL13	Fe	<i>FRO1</i>	At1g01590
CVL14	Fe	<i>Nramp3</i>	At2g23150	CL13	Fe	<i>FRO2</i>	At1g01580
CVL58	P	<i>Pht1;4</i>	At2g38940	CVL16	Fe	<i>FRO4</i>	At5g23980
CVL33	P	<i>Pht1;5</i>	At2g32830	CVL16	Fe	<i>FRO5</i>	At5g23990
CL37	P	<i>Pht1;9</i>	At1g76430	CL19	Fe	<i>FRO6</i>	At5g49730
CVL38	S	<i>Sultr1;2</i>	At1g78000	CL19	Fe	<i>FRO7</i>	At5g49740
CVL38	S	<i>Sultr2;2</i>	At1g77990	CL19	Fe	<i>FRO8</i>	At5g50160
CL40	S	<i>Sultr3;4</i>	At1g23090	CL15	Fe	<i>NFU1</i>	At4g01940
CVL60	S	<i>Sultr3;3</i>	At3g15990				
CVL39, CVL61	S	<i>Sultr4;2</i>	At3g12520				
CVL16, CVL47	Fe	<i>YSL2</i>	At5g24380				
CL12	Cu	<i>YSL3</i>	At5g53550				
CVL7, CVL50	Cu, Zn	<i>YSL7</i>	At1g65730				
CL46	Zn	<i>YSL8</i>	At1g48370				
CL48, CVL47	Zn	<i>ZIF1</i>	At5g13740				
CL48, CVL47	Zn	<i>ZIFL1</i>	At5g13750				
CVL46	Zn	<i>ZIP</i> family	At3g08650				
CL14	Zn	<i>ZIP</i> family	At3g08650				
CVL46	Zn	<i>ZIP1</i>	At3g12750				
CVL49	Zn	<i>ZIP2</i>	At5g59520				
CVL45	Zn	<i>ZIP3</i>	At2g32270				
CVL44	Zn	<i>ZIP4</i>	At1g10970				
CVL44	Zn	<i>ZIP5</i>	At1g05300				
CL47, CVL45	Zn	<i>ZIP6</i>	At2g30080				
CVL49	Zn	<i>ZIP12</i>	At5g62160				

*AKT*, Arabidopsis potassium transporter; *APR*, APS reductase; *APS*, ATP sulfurylase; *ATX*, anti-oxidant; *CAX*, cation exchanger; *CCH*, copper chaperone; *COPT*, copper transporter; *CSD*, Cu, Zn superoxide dismutase; *CXIP*, CAX-interacting protein; *FER*, ferritin; *FRD*, ferric reductase defective; *FRO*, ferric reductase oxidase; *HMA*, heavy metal P1b-ATPase; *KT*, potassium transporter; *KUP*, potassium uptake permease; *MGT*, magnesium transporter; *MRS*, mitochondrial RNA splicing; *MT*, metallothioneine; *MTP*, metal tolerance protein; *NFU*, nitrogen-fixation-specific-like; *Nramp*, natural resistance-associated macrophage protein; *PAA*, P-type ATPase of Arabidopsis; *PHO*, phosphate overaccumulator; *Pht*, phosphate transporter; *RAN*, responsive to antagonist; *Sultr*, sulfate transporter; *YSL*, yellow stripe-like; *ZIF*, zinc-induced facilitator; *ZIFL*, ZIF-like; *ZIP*, zinc regulated transporter, iron regulated transporter-like protein.

**Table 6** Relative mineral concentrations of wild-type and *erecta* (*er*) mutant seeds of *Arabidopsis thaliana* grown under high light, high nutrients (HLHN), low light, low nutrients (LLN), or low light, high nutrients (LLHN; two experiments shown)

Treatment	Line	Ca	Cu	Fe	K	Mg	Mn	P	S	Zn
HLHN	Col	100	100	100	100	100	100	100	100	100
	Ler <sup>a</sup>	72	119	138	95	96	102	75	85	119
	<i>er</i> -114 <sup>a</sup>	87*	80*	89	110	91*	79*	87*	90*	98
	<i>er</i> -116 <sup>a</sup>	86*	84*	100	111*	94*	93	95	80*	99
	<i>er</i> -117 <sup>a</sup>	91	74*	91	102	95	88*	94	91	97
	Ws-2	100	100	100	100	100	100	100	100	100
	<i>er</i> -123 <sup>b</sup>	97	176*	106	170*	115*	92*	146*	92	134*
LLN	Col	100	100	100	100	100	100	100	100	100
	Ler <sup>a</sup>	103	201	134	81	90	132	83	99	138
	<i>er</i> -114 <sup>a</sup>	143*	210*	139	100	99	86	115	124	131
	<i>er</i> -116 <sup>a</sup>	119*	114*	81*	108	99	92*	113	95	106*
	<i>er</i> -117 <sup>a</sup>	140*	155*	115	130*	100	78*	115	111	113*
	Ws-2	100	100	100	100	100	100	100	100	100
	<i>er</i> -123 <sup>b</sup>	113*	155*	72	147*	121*	71*	139*	112	114*
LLHN (Expt 1)	Col	100	100	100	100	100	100	100	100	100
	Ler <sup>a</sup>	84	146	138	66	93	159	69	99	155
	<i>er</i> -114 <sup>a</sup>	121*	144*	123*	91*	104	125*	108	113	140*
	<i>er</i> -116 <sup>a</sup>	102	102	95	91	98	125*	94	99	117*
	<i>er</i> -117 <sup>a</sup>	111*	124*	97	106*	103	100	104	108	124*
	Ws-2	100	100	100	100	100	100	100	100	100
	<i>er</i> -123 <sup>b</sup>	117*	146*	109*	116	103	73*	115*	115*	107*
LLHN (Expt 2)	Col	100	100	100	100	100	100	100	100	100
	Ler <sup>a</sup>	85	165	186	92	99	140	92	107	184
	<i>er</i> -114 <sup>a</sup>	89*	105	172*	106*	118*	97	100	122*	158*
	<i>er</i> -116 <sup>a</sup>	91	109	147*	136*	121*	98	110*	116*	166*
	<i>er</i> -117 <sup>a</sup>	117*	93	143*	110*	107*	82*	97	111*	144*
	Ws-2	100	100	100	100	100	100	100	100	100
	<i>er</i> -123 <sup>b</sup>	110	124*	111*	138*	120*	85*	118*	102	121*

\*Significantly different than wild type ( $P < 0.05$ ).<sup>a</sup>Normalized to Columbia (Col).<sup>b</sup>Normalized to Wassilewskija-2 (Ws-2).

that encode regulatory proteins. Genes that encode proteins vital to these processes are very likely to also be important for the orthologous processes in crop plants. Thus, discovery of genes that increase *A. thaliana* seed mineral concentrations could help to determine the most effective genes to target for biofortification of crops.

### Robustness of traits

One of the main objectives of this research was to determine whether *A. thaliana* is a reliable model plant for QTL mapping of seed mineral concentrations for discovery of target genes for biofortification. To meet this criterion, significant QTLs should be robust enough to be mapped in multiple growth cycles and, ideally, in similar but differing environments, such as growth facilities of different laboratories or different seasons within a given growth facility. Thus, we thought it important to compare our results with those from

another research group (Vreugdenhil *et al.*, 2004). On each occasion on which the RIL populations were grown, there was a wide range of values (2–3-fold) observed for each trait (Table 1), and a normal distribution of the traits within these ranges (Fig. 1, Supporting Information Figs S1–S3), indicating that adequate trait diversity accompanies the natural genetic variation. A wide range of mineral concentration values has been observed in edible portions among accessions of a number of crop plants (reviewed by White & Broadley, 2005).

Between occasions of RIL population growth, most minerals that were correlated on one occasion were similarly correlated on other occasions (Fig. 2, Supporting Information Table S1), further indicating that *A. thaliana* seed mineral concentrations are reliable traits. The most important test, however, is repetition of the QTL mapped for each trait. If seed mineral QTLs are repeated between different research groups and different experiments within a group, that is a good indication that the quantitative gene effect may be sufficiently robust to pursue by fine

mapping or a candidate gene approach. Several seed mineral QTLs met this criteria; four QTLs in the CL population and five QTLs in the CVL population were identified in three separate experiments, while another 15 QTLs were identified in two of three experiments. It would be useful to grow the RIL populations in differing environments, such as with low nutrient supply or higher light intensity, to see if the seed mineral concentration QTLs are maintained over more varied environments.

### Linking genetics to physiology

While the bulk of mineral nutrients in *A. thaliana* seeds come into the plant during seed fill (Waters & Grusak, 2008), differences in translocation through the plant or remobilization from source tissues may account for the small differences between RILs. Because some minerals have been shown to be mobilized from *A. thaliana* silique walls (hulls) to seeds during fruit maturation, and the mineral concentration of mature hulls is reflective of the mineral content of hulls (Waters & Grusak, 2008), the seed:hull concentration ratio can be considered a quantitative trait that reflects fruit mineral partitioning to seeds. Genes important for remobilization or translocation of minerals through hull tissue may be specific to this tissue, but could also be important for these processes in other seed mineral source tissues, such as leaves. Seven hull QTLs were also mapped from seed mineral concentrations, and four of the six QTLs for seed:hull concentration ratios were also found in hull or seed mineral concentration QTLs, indicating that differences in mineral partitioning between lines can also be used as a quantitative trait.

The strongest seed mineral QTL identified in this study, for P at the top of chromosome 3 in the CVL population, was previously identified (Bentsink *et al.*, 2003; Vreugdenhil *et al.*, 2004). In our study, this QTL was found not only for seed P in both CVL experiments, but also for P in silique hulls and the seed:hull ratio. This indicates that the quantitative trait gene is robust enough to affect and be detected for multiple traits, highlighting the importance of comparing multiple experiments, and the potential for performing QTL analysis on certain source tissues that might affect the ultimate trait of interest. Bentsink *et al.* (2003) have initiated fine mapping of this region of chromosome 3.

Although a number of QTLs were identified in multiple experiments, most of the QTLs mapped were found in only one experiment. It is unclear why this was the case, but a number of explanations can be offered. First, most of the single-occurrence QTLs had small effects on seed mineral traits and significant but low LR scores. Thus, these loci may in fact have a minor effect on seed mineral concentration, but, as a result of slightly varying environmental conditions between experiments, may not have been as important in each experiment. If this is the case, it would be impractical to fine map these weak QTLs. Secondly, these weak QTLs may reflect the quantitative nature of seed mineral traits, where dozens of

gene products may have small additive effects on the processes involved in mineral movement to and accumulation in seeds, and in a given replicate experiment these weak QTLs were not always statistically significant. Thirdly, another reason why individual QTLs may be weak or not mapped in replicate experiments could be that many genes involved in plant mineral nutrition are members of gene families with individual members overlapping in function and expression patterns. It is not uncommon for single-gene knockouts to have no detectable mutant phenotype (as we observed with *hma5*, *ysl8*, and *zip5*), whereas disruption of two gene family members results in a severe mutant phenotype (Hussain *et al.*, 2004; Waters *et al.*, 2006).

### Candidate genes

Genes related to mineral transport or homeostasis (or annotated as such) that fell within QTL confidence intervals are listed in Table 5. While we realize that the majority of mineral-related genes in the QTL region will not be the quantitative trait gene detected within that confidence interval, it is logical to consider the potential of these genes to have an effect on seed mineral concentration. Most of these genes were categorized as transporters, while others fell into the categories of chelators and storage molecules, regulators of transporters or homeostasis, or genes involved in metabolism. Many of the candidate genes are members of gene families. The ultimate confirmation of genes responsible for each of the seed traits will be challenging, but may include evidence such as that obtained from positional cloning by construction of near isogenic lines (NILs), association mapping of specific polymorphisms, transgenic or deficiency complementation, gene expression analysis, or mutational analysis (Koornneef *et al.*, 2004; Weigel & Nordborg, 2005).

On chromosome 2, several of the QTLs mapped to a region that coincided with the *ER* locus, with additive effects indicating that the *er* mutant lines had higher concentrations of Ca, Cu, K, Mg, Mn, P, S, and Zn in the CL population, and Cu, Fe, Mg, P, and Zn in the CVL population. Recently, the *ER* locus was shown to affect water use efficiency (Masle *et al.*, 2005), which may in turn differentially affect mineral translocation in the xylem to aerial parts of the plant. We tested the idea that *er* might affect seed mineral concentration using single *er* mutants, and based on the results (Table 6), it appears that Ca, Cu, and Zn are consistently increased in most of the *er* mutant lines in low-light conditions, although penetrance was not 100%. Results were mixed for Fe, K, and Mg, while no consistent increase in seed mineral concentration was observed in *er* mutants for Mn, P, and S. These results suggest that for Mn, P and S QTLs in this region of chromosome 2, some gene other than *er* is responsible for the seed mineral variation. However, it appears likely that the *er* locus itself contributes to increasing seed mineral Ca, Cu and Zn concentrations under low-light conditions, but not under high-light conditions

where increased stomatal conductance to support higher photosynthetic rates would result in increased transpiration both in *er* and in *ER* plants, thereby minimizing water flux differences between mutant and wild-type lines. Conversely, when environmental conditions lead to differential rates of transpiration in *ER* versus *er* lines and less overall transpiration in *ER* lines (e.g. lower light), reduced xylem flow, especially to low-transpiring organs such as siliques (Jensen *et al.*, 1998), may lead to lowered delivery of minerals to these structures. Because silique hulls are an important source tissue for seed minerals (Waters & Grusak, 2008), a reduced delivery of minerals to the silique hulls (*ER* relative to *er* lines) could explain the reduced seed concentrations of certain minerals. However, as the *ER* gene encodes a serine/threonine protein kinase and the *er* mutation has pleiotropic effects (Lease *et al.*, 2001), reasons other than water use efficiency, including interactions with other genes, could affect seed mineral concentrations. In the RIL populations, the effects of the QTLs containing *er* could be explained by other nearby genes; there are Cu- and Zn-related genes in this region that could be involved in metal translocation within the plant.

#### Candidate genes: Cu, Fe, and Zn

Several members of the copper transporter (COPT) family of Cu transporters are found in seed and hull Cu-concentration QTL regions (Table 5). *COPT* family genes are expressed in roots, leaves, stems, and flower tissues (Sancenón *et al.*, 2003). *COPT1* is the most well-characterized family member. *COPT1* antisense lines had 40–60% lower Cu concentrations in rosette leaves and defective pollen development (Sancenón *et al.*, 2004).

Four members of the heavy metal P1b-ATPase (*HMA*) family are located within seed and hull mineral QTL confidence intervals. *HMA4* was in Zn seed and hull concentration QTL confidence intervals. Rosette Zn concentration is low in *hma4* mutants (Baxter *et al.*, 2007), and was important for translocation of Zn from roots to shoots (Hussain *et al.*, 2004; Verret *et al.*, 2004). *HMA5*, *HMA7* and *HMA8* are monovalent Cu transporters. *HMA5* is found in two Cu QTLs, one for seed concentration and one for hull concentration. Mutant *hma5* plants grown on high Cu had increased Cu concentration mainly in the roots (Andrés-Colás *et al.*, 2006). *HMA7* response to antagonist 1 (*RAN1*) is necessary for loading Cu into intracellular compartments of the secretory pathway (Hirayama *et al.*, 1999). *HMA8*/P-type ATPase of Arabidopsis 2 (*PAA2*) is required for Cu transport into thylakoids and delivery to plastocyanin (Abdel-Ghany *et al.*, 2005); *hma8/paa2* mutants had increased expression of stroma-localized Cu, Zn superoxide dismutase *CSD2* (Abdel-Ghany *et al.*, 2005). *CSD2* and *CSD3* are both localized to QTLs mapped for seed Zn concentration and for seed and hull Cu concentration (Table 5).

Within cells, Cu is trafficked bound to chaperone proteins, such as copper chaperone (CCH) and anti-oxidant 1 (ATX1).

*CCH*, *ATX1*, and three genes annotated as *CCH-like* are all within Cu QTL regions. *ATX1* interacts with *HMA5* (Andrés-Colás *et al.*, 2006) and with *HMA7/RAN1* (Puig *et al.*, 2007). *CCH* and *HMA7/RAN1* are both up-regulated during leaf senescence (Himelblau & Amasino, 2000; Mira *et al.*, 2001) and could be important for removing Cu from leaves to be translocated to seeds. Up to 29% of total seed Cu content was derived from vegetative tissue remobilization in *A. thaliana* (Waters & Grusak, 2008). Several genes related to Cu homeostasis were differentially regulated in *A. thaliana* lines differing in Cu tolerance and Cu accumulation, including *Col* and *Ler* (Schiavon *et al.*, 2007), lending credence to the idea that differences in the expression or sequence of these genes could alter quantitative traits such as seed mineral concentration.

*YSL* family genes encode metal-nicotianamine transporters (DiDonato *et al.*, 2004; Koike *et al.*, 2004; Schaaf *et al.*, 2005). Several members of the *YSL* family are located within seed mineral QTL regions for Fe and Zn. Mutations in *YSL1* and *YSL3* resulted in decreased seed concentrations of Fe, Zn, and Cu (Le Jean *et al.*, 2005; Waters *et al.*, 2006; Waters & Grusak, 2008), while *ysl1ysl3* mutants overaccumulated Cu and Zn in leaves (Waters & Grusak, 2008).

An uncharacterized cation diffusion facilitator metal tolerance protein (*MTP*)-family gene, *MTPB1*, is located within a seed Zn-concentration QTL. *Arabidopsis thaliana* mutants of the vacuole-localized *mtp1* had lower accumulation of Zn in stem and leaf tissue, but normal Zn concentration in flowers and siliques (Desbrosses-Fonrouge *et al.*, 2005), whereas *MTP3* moves Zn into vacuoles in Fe-deficient plants and is important for regulating Zn partitioning between roots and shoots (Arrivault *et al.*, 2006). Another family of Zn transporters, the ZIP proteins, are responsible for Zn uptake from the soil, and some members that are expressed in shoot tissues (Grotz *et al.*, 1998) may be involved in translocation of Zn throughout the plant (Colangelo & Guerinot, 2006). Several ZIP family members are localized in QTL regions for Fe and Zn (Table 5).

The Fe-citrate transporter ferric reductase defective 3 (*FRD3*) (Durrett *et al.*, 2007) controls root-to-shoot Fe translocation (Green & Rogers, 2004), and localizes to a seed Fe QTL, as does an annotated gene similar to zebrafish ferroportin (Donovan *et al.*, 2000), which is an Fe exporter in the gut. An analogous function in plants may be to move Fe into the apoplasm for loading into the xylem pathway. Before internal translocation, Fe uptake from the soil depends on reduction by ferric reductase oxidase 2 (*FRO2*; Robinson *et al.*, 1999). The *FRO2* gene and several other *FRO* family members that are expressed in leaf vascular and flower tissues (Wu *et al.*, 2005; Mukherjee *et al.*, 2006) are found in three seed Fe QTL regions.

Mineral hyperaccumulating plants can have mineral concentrations several orders of magnitude greater than crop plants and often have higher expression of mineral transporter genes than their nonhyperaccumulating relatives (Broadley *et al.*, 2007). The widely studied Zn hyperaccumulator *Thlaspi*

*caerulescens* can accumulate high concentrations of Zn not only in vegetative tissues, but also in seeds (Ernst, 1996). Identification of genes that contribute to this trait may offer clues as to which genes would make effective biofortification targets in crop plants. Several microarray studies identified genes more highly expressed in the Zn hyperaccumulator *Arabidopsis halleri* than in *A. thaliana*, and revealed several genes identical to or in the same families as our candidate genes, including genes of the *HMA* family, the *MTP* family, natural resistance-associated macrophage protein 3 (*Nramp3*), *YSL6*, *FRD3*, and several *ZIP* family members (Becher *et al.*, 2004; Weber *et al.*, 2004; Filatov *et al.*, 2006; Talke *et al.*, 2006; Broadley *et al.*, 2007). Similarly, genes more highly expressed in *T. caerulescens* than in *A. thaliana* included *Nramp3*, *HMA* family genes, *ZIP* family genes, *YSL7*, *MTP1*, *FRD3*, and *FRO5* (van de Mortel *et al.*, 2006).

### Candidate genes: P and S

The phosphate transporter 1 (*Pht1*) family encodes phosphate transporter proteins, and several *Pht1* genes are localized in QTL regions. *Pht1;4* is one of these, and has been demonstrated to be important for uptake of inorganic phosphate (P<sub>i</sub>) into the root (Muchhal *et al.*, 1996; Misson *et al.*, 2004; Shin *et al.*, 2004), whereas work using a *Pht1;5* promoter-reporter construct indicated that expression was primarily in leaves (Mudge *et al.*, 2002). The finding that phosphate overaccumulator 2 (*pho2*) mutants had 3-fold higher P concentrations in shoots, and higher P in stems, siliques, and seeds suggests that PHO2 is a regulator of P accumulation (Delhaize & Randall, 1995).

Five members of the sulfate transporter (*Sultr*) family of sulfate transporters are localized in QTL regions for seed and hull S concentration, three in plasma membrane-localized subgroups 1–3, and one in vacuole-localized subgroup 4 (Hawkesford & De Kok, 2006). Of these candidate genes, *Sultr1;2* has been indicated to be important for root sulfate uptake (Shibagaki *et al.*, 2002), while *Sultr2;2* is a low-affinity transporter expressed near vascular tissue in roots and leaves (Takahashi *et al.*, 2000). Other family members have been implicated in redistribution of S from source tissues (Yoshimoto *et al.*, 2003), and loss of *Sultr2;1* function resulted in decreased seed S concentration (Awazuhara *et al.*, 2005). Other candidate genes are involved in S metabolism (Hawkesford & De Kok, 2006), including ATP sulfurylase 1 (*APS1*), APS kinase, and APS reductase 1 (*APR1*).

### Conclusions

Candidate genes were identified for many of the QTLs based on known or predicted gene function, but other QTLs have no obvious candidates, suggesting that new genes affecting mineral uptake or translocation are yet to be identified. Many QTLs were identified for seed mineral concentration traits in two *A. thaliana* RIL populations, with several of these QTLs found in multiple experiments, and thus are sufficiently

robust to allow future fine mapping of the quantitative genes. This suggests that *A. thaliana* is a reliable model for discovery of genes that could be targeted for use in biofortification efforts to increase mineral concentrations in seeds of crop plants. The fact that the majority of QTLs were identified in only one experiment suggests that seed mineral concentration is a truly quantitative trait; many genes have small additive effects on the final trait value. This indicates that, to develop biofortified crops, several genes may need to be targeted simultaneously, highlighting the importance of developing our understanding of the molecular and physiological steps required to accumulate minerals in seeds.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Histograms of seed mineral concentration frequency distributions in Col × *Ler* recombinant inbred line (RIL) populations.

**Fig. S2** Histograms of seed mineral concentration frequency distributions in Cvi × *Ler* recombinant inbred line (RIL) populations.

**Fig. S3** Histograms of silique hull mineral concentrations in Cvi × *Ler* recombinant inbred line (RIL) populations.

**Fig. S4** Correlation between seed mineral concentration of individual recombinant inbred lines (RILs) in 2003 and 2005 or 2007 in the Col × *Ler* population.

**Fig. S5** Correlation between seed mineral concentration of individual recombinant inbred lines (RILs) in 2005 and 2007 in the Col × *Ler* population.

**Fig. S6** Correlation between seed mineral concentration of individual recombinant inbred lines (RILs) in 2003 and 2006 in the Cvi × *Ler* population.

**Fig. S7** Genetic map and quantitative trait locus (QTL) positions for seed mineral concentrations in 2003, 2005, and 2007 for the Col × *Ler* population.

**Fig. S8** Genetic map and quantitative trait locus (QTL) positions for seed mineral concentrations in 2003 and 2006 for the Cvi × *Ler* population.

**Fig. S9** Genetic map and quantitative trait locus (QTL) positions for silique hull and seed:hull mineral concentrations in 2003 and 2006 for the Cvi  $\times$  Ler population.

**Table S1** Significance thresholds (likelihood ratio (LR) score) for  $P < 0.05$  for composite interval mapping, as determined by 1000 permutations of the data

**Table S2** Correlations between minerals by experiment

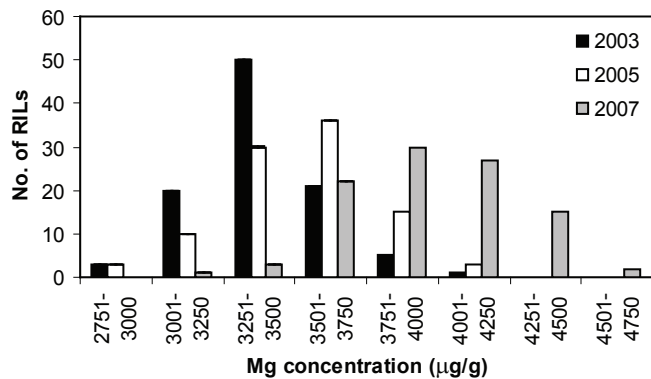
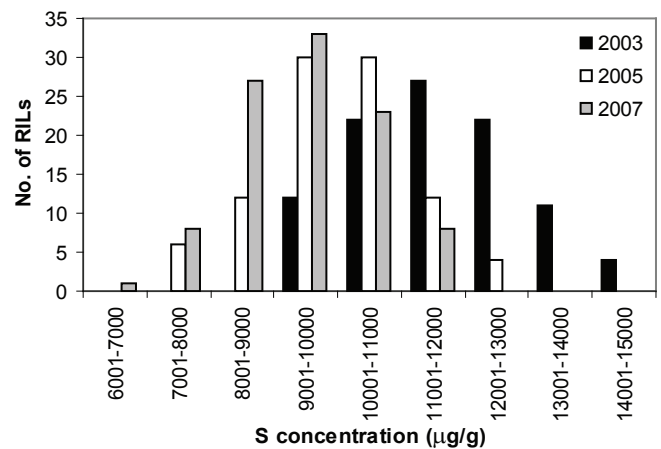
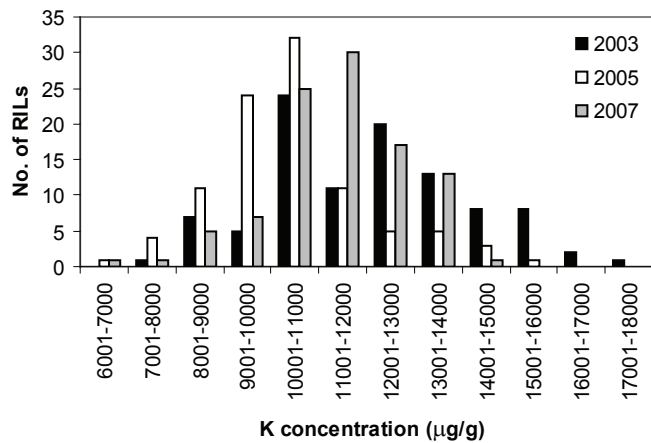
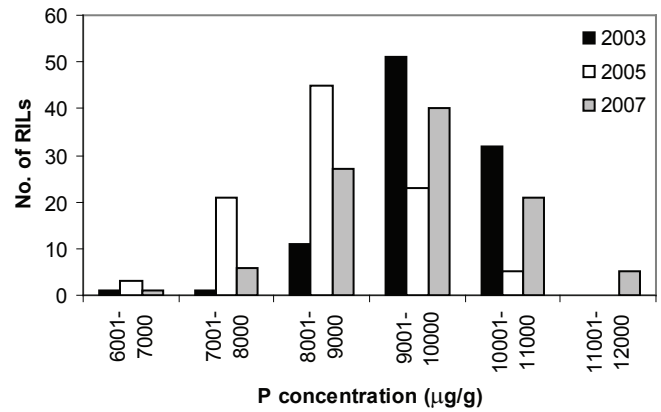
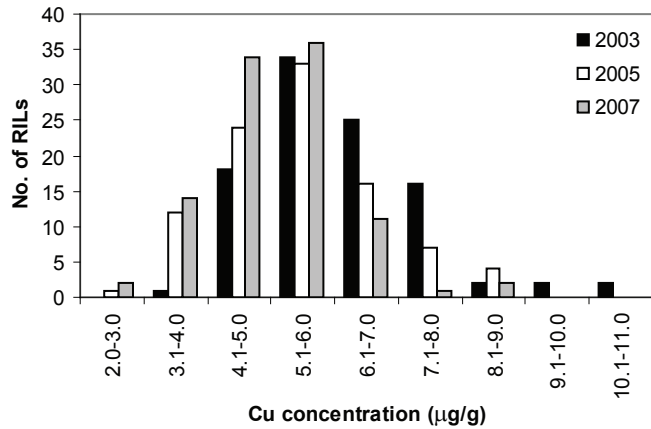
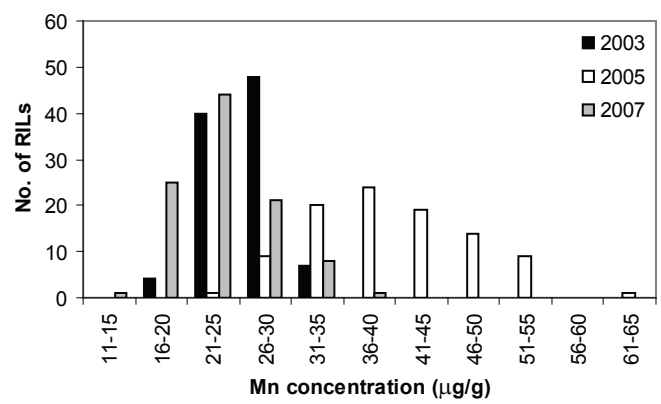
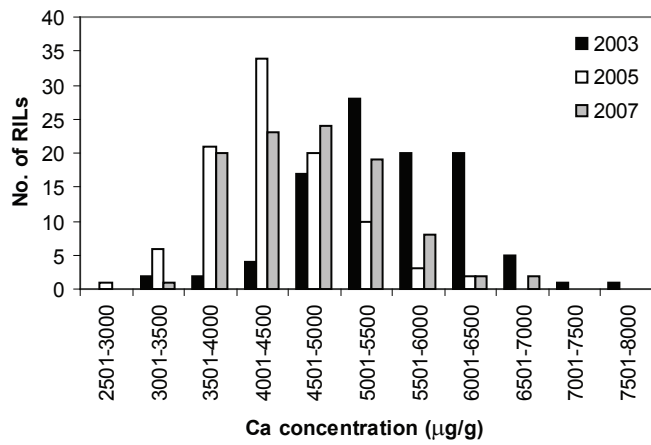
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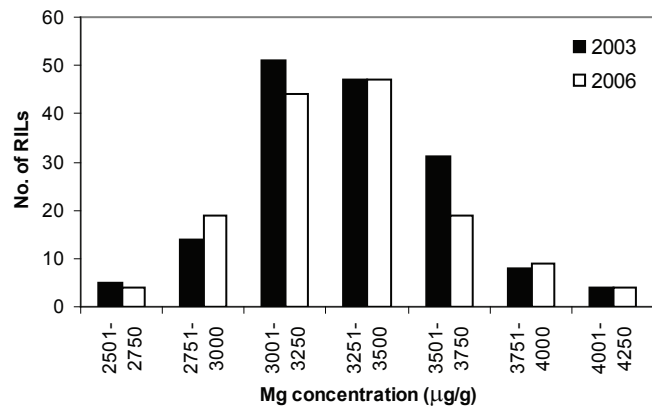
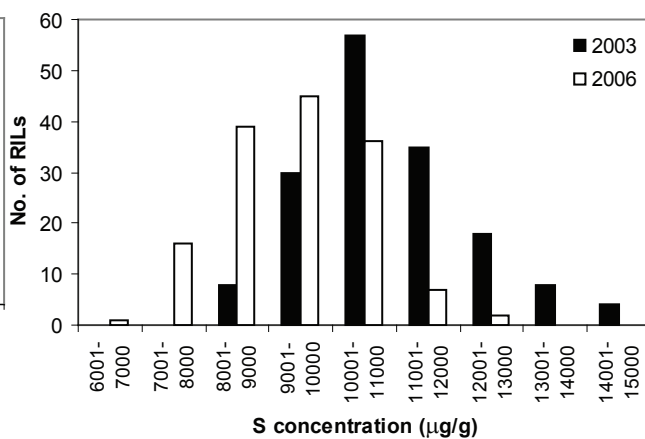
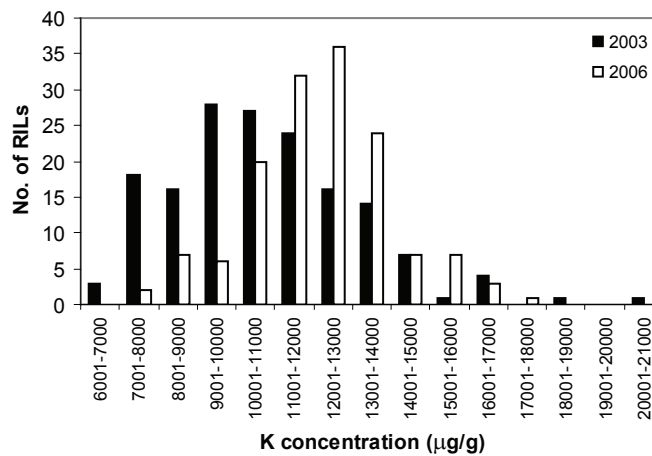
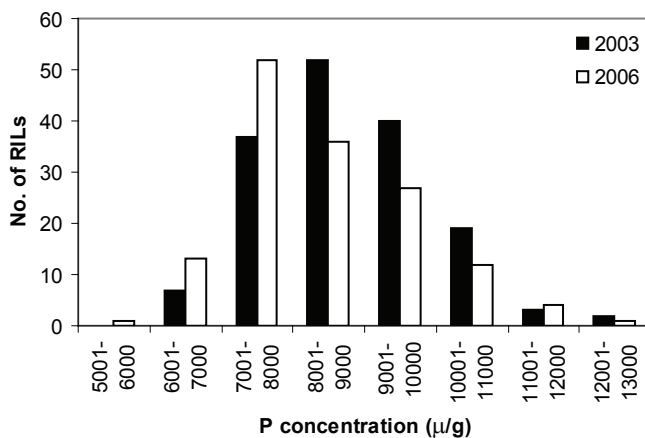
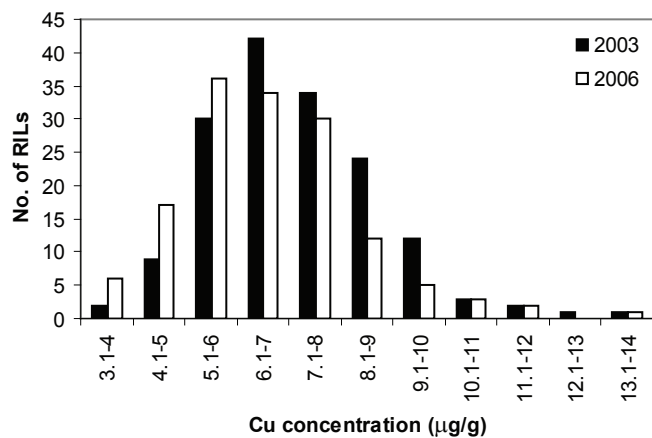
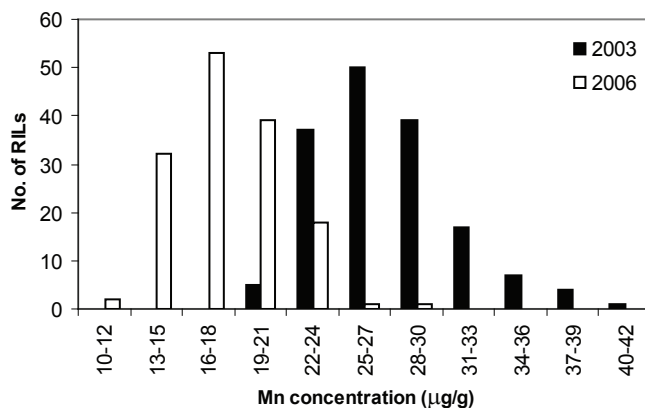
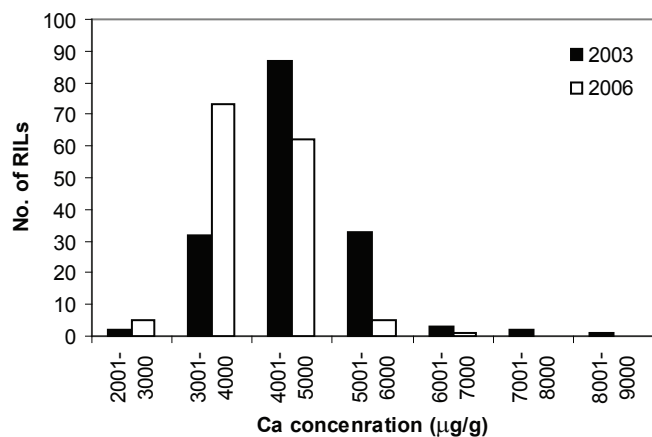
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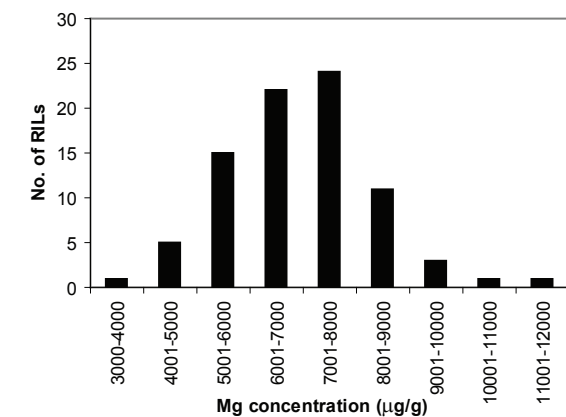
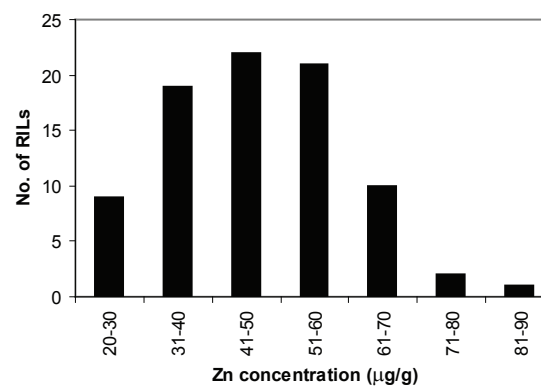
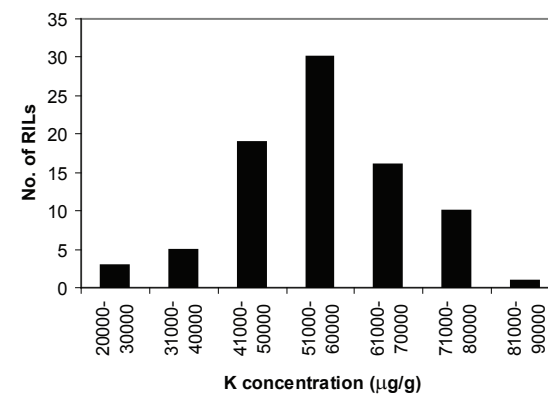
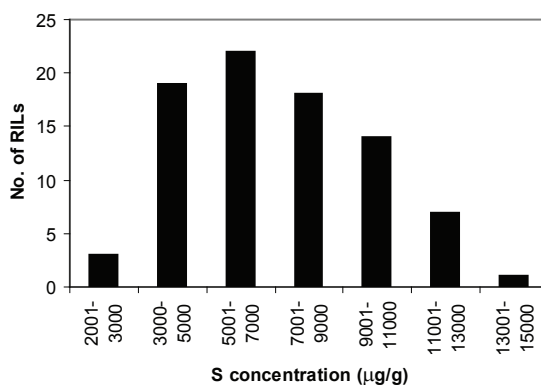
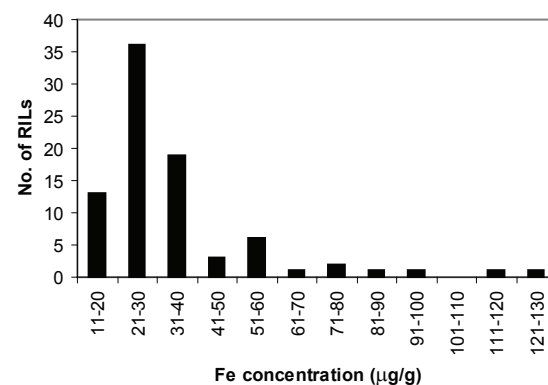
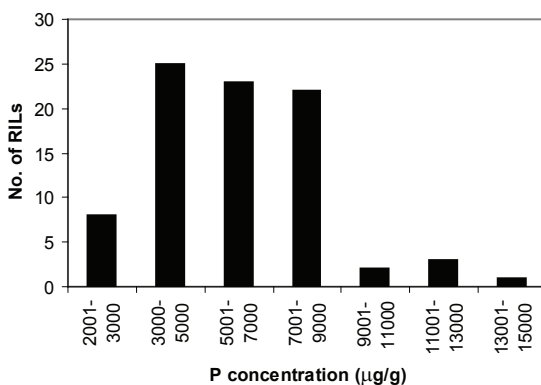
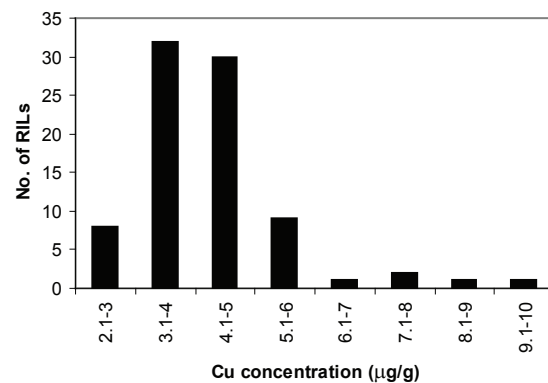
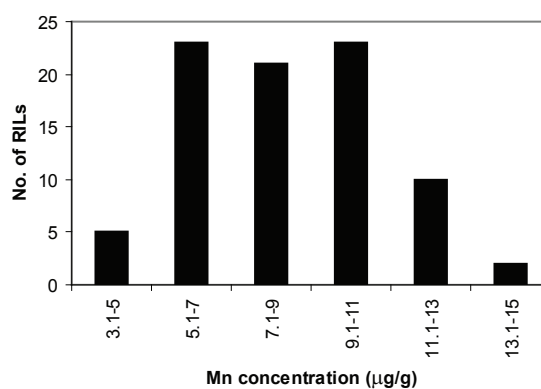
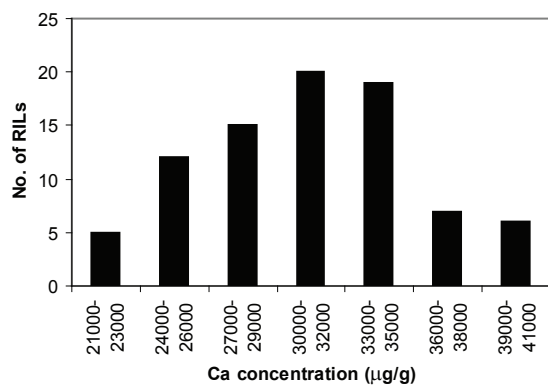




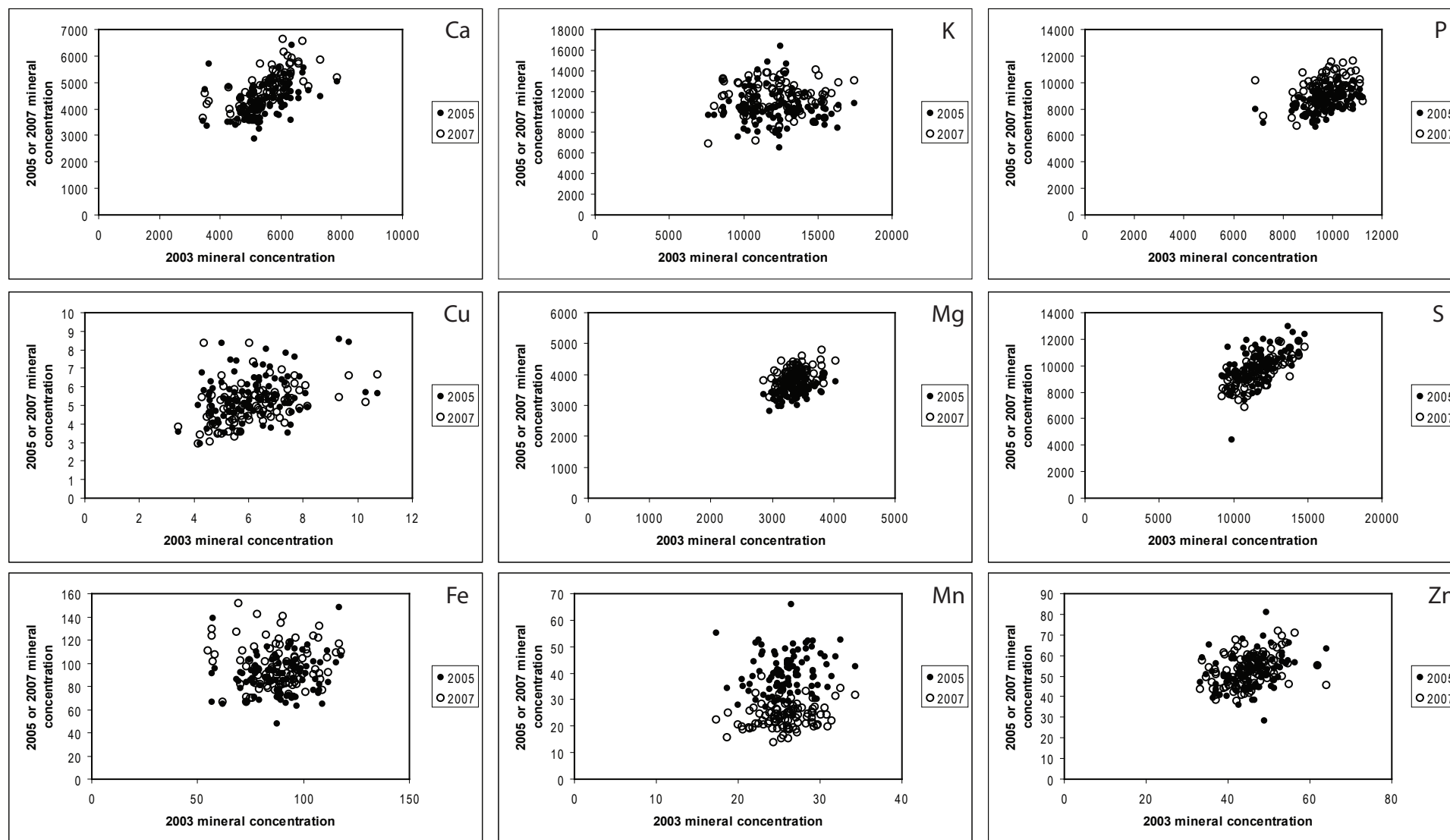
Suppl. Fig. 1. Histograms of seed mineral concentration frequency distributions in Col x Ler RIL populations.



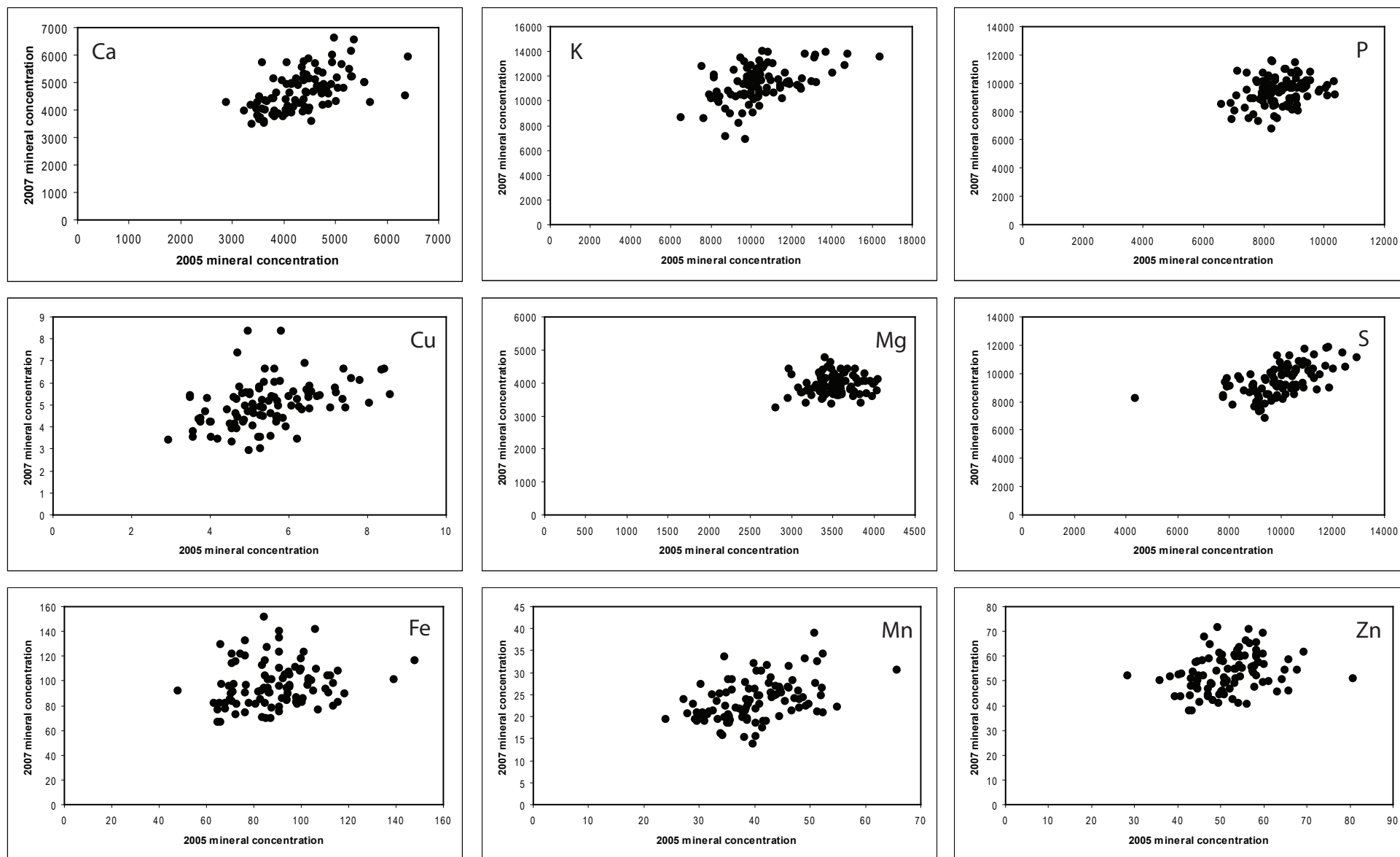
Suppl. Fig. 2. Histograms of seed mineral concentration frequency distributions in Cvi x Ler RIL populations.



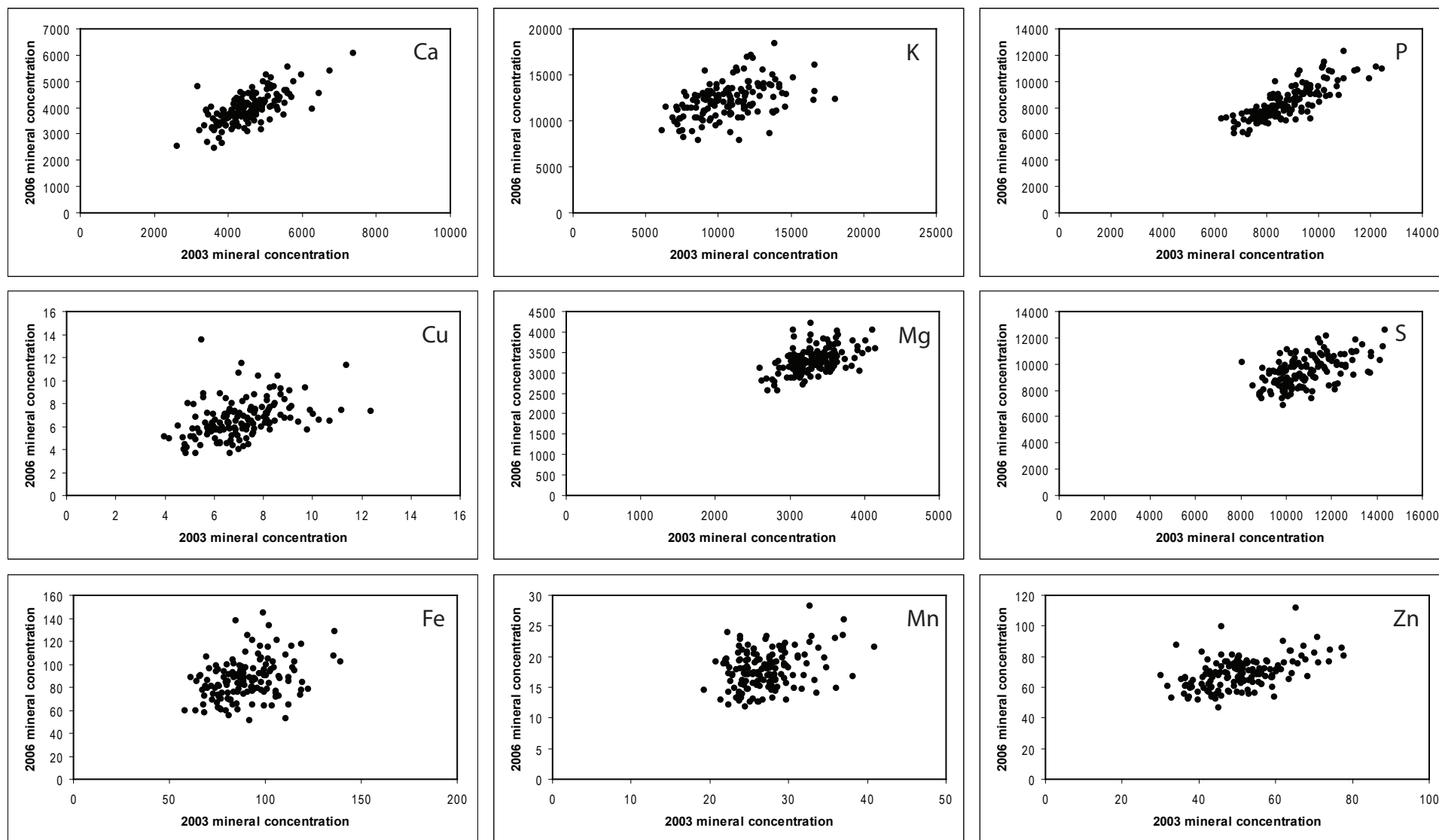
Suppl. Fig. 3. Histograms of silique hull mineral concentration frequency distributions in 2006 Cvi x Ler RIL population.



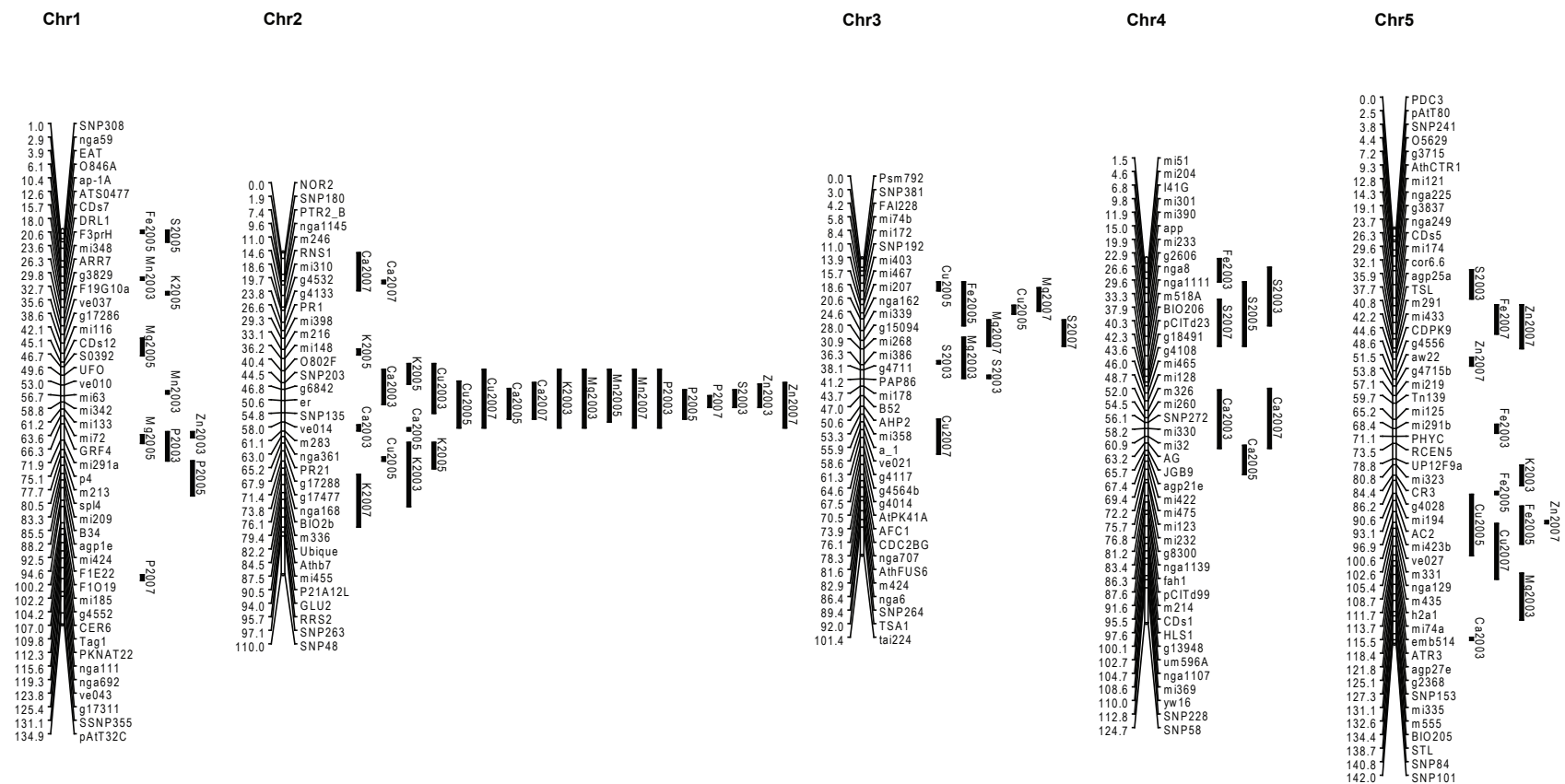
Supplemental Figure 4. Correlation between seed mineral concentrations of individual RILs in 2003 and 2005 or 2007 for Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn in the Col x Ler RIL population.



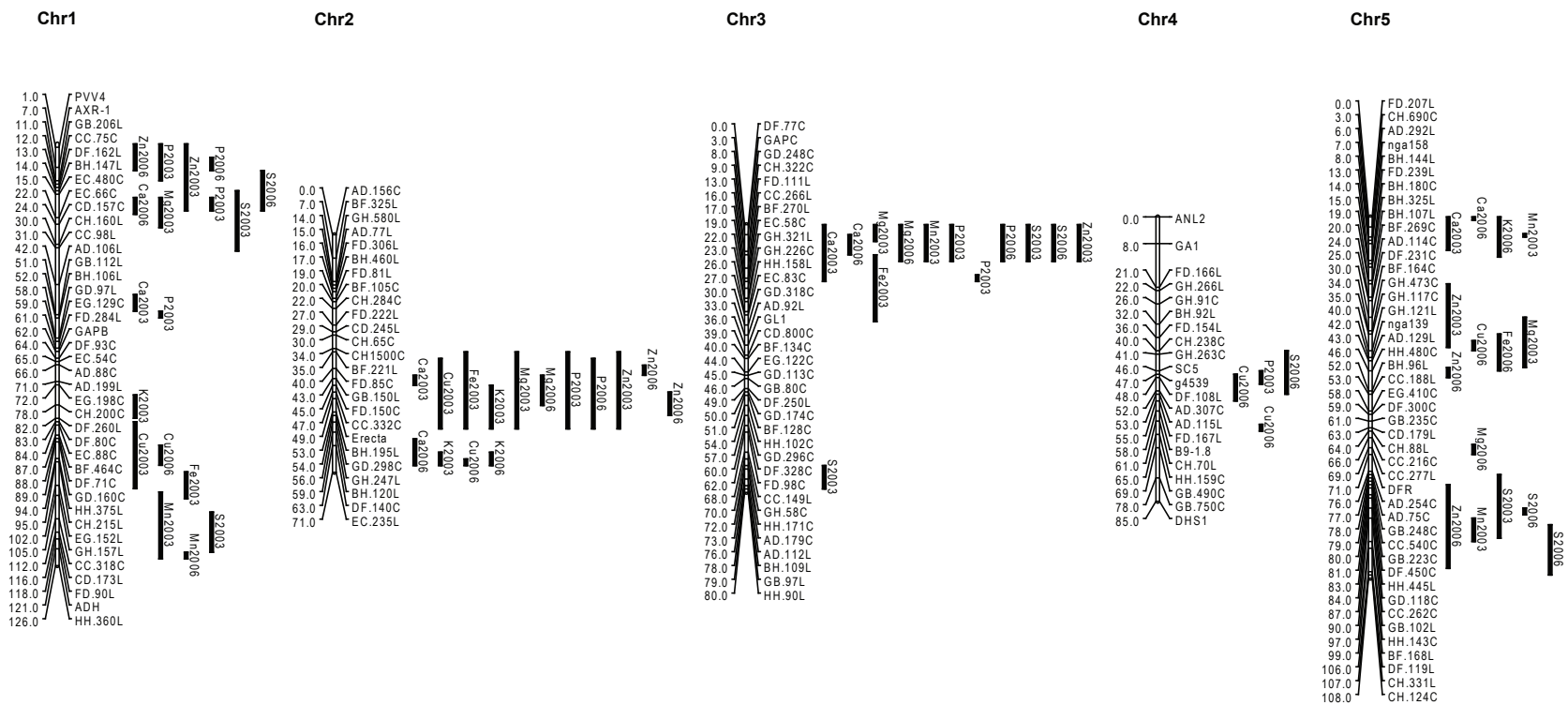
Supplemental Figure 5. Correlation between seed mineral concentrations of individual RILs in 2005 and 2007 for Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn in the Col x Ler RIL population.



Supplemental Figure 6. Correlation between seed mineral concentrations of individual RILs in 2003 and 2006 for Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn in the Cvi x Ler RIL population.

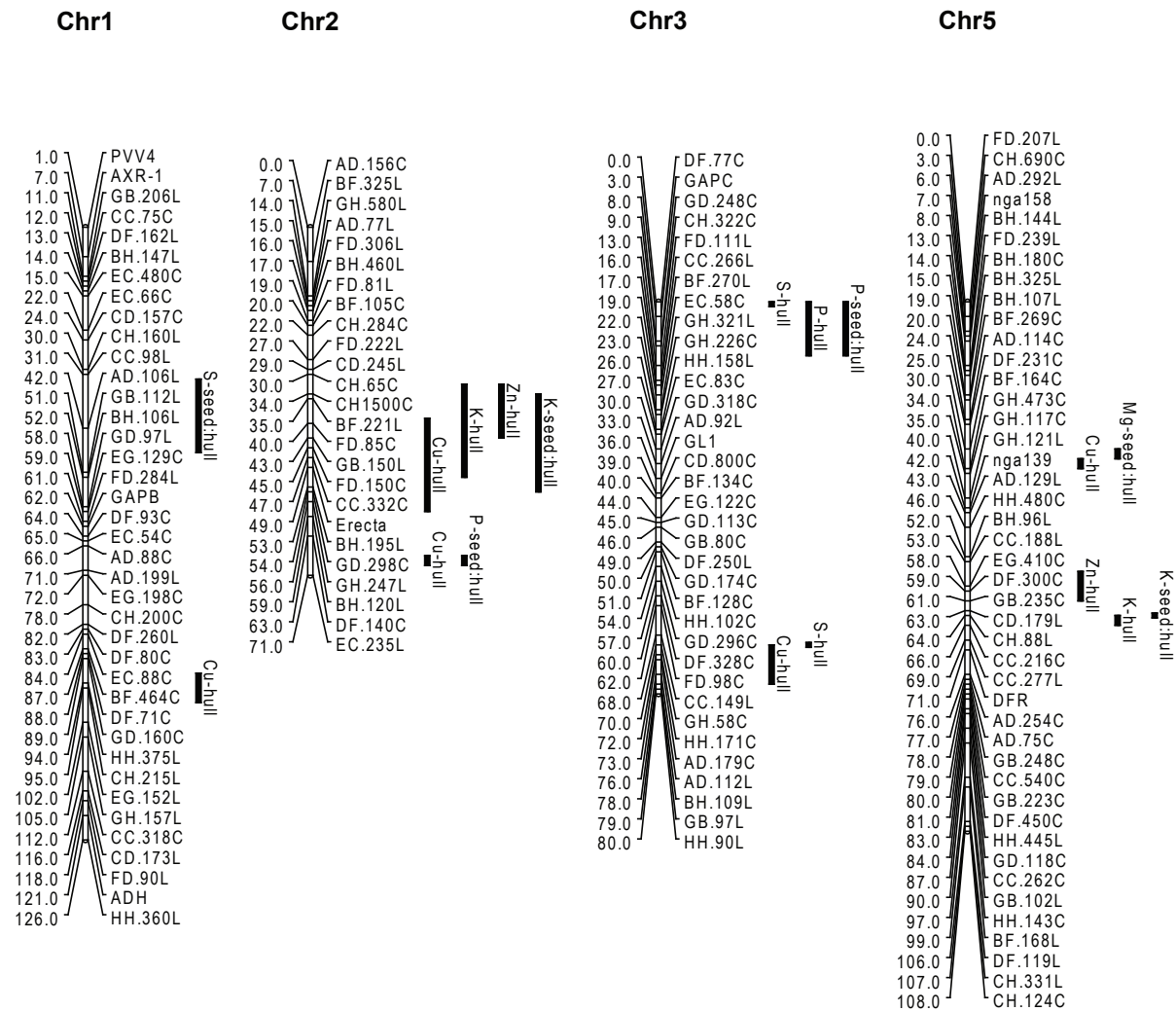


Supplemental Figure 7. Genetic map and QTL positions for seed mineral concentrations in 2003, 2005, and 2007 for the Col x Ler population. Length of bar represents confidence interval.



Supplemental Figure 8. Genetic map and QTL positions for seed mineral concentrations in 2003 and 2006 for the Cvi x Ler population. Length of bar represents confidence interval.





Supplemental Figure 9. Genetic map and QTL positions for silique hull and seed:hull mineral concentrations in 2003 and 2006 for the Cvi x Ler population. Length of bar represents confidence interval.

**Supplementary material Table S1** Significance thresholds (LR score) for  $P < 0.05$  for composite interval mapping, as determined by 1000 permutations of the data. ns, no significant QTL.

Trait	Experiment						
	CVL 2003	CVL 2006	CVL hulls	CVL seed:hull ratio	CL 2003	CL2005	CL2007
Ca	11.3	12.4	ns	ns	12.6	12.3	13.1
Cu	12.2	11.9	10.9	ns	12.7	12.1	12.7
Fe	12.0	7.4	ns	ns	13.0	12.2	12.4
K	11.6	12.2	12.2	12.1	12.8	12.3	12.2
Mg	12.3	12.0	ns	11.7	12.5	12.6	12.2
Mn	12.2	12.5	ns	ns	12.5	12.3	12.1
P	11.9	12.1	11.9	12.0	12.2	12.5	13.0
S	11.5	12.5	11.8	12.0	11.5	11.5	11.5
Zn	12.2	11.9	12.5	ns	12.3	ns	12.9

**Supplementary material Table S2** Correlations between minerals by experiment. Correlations are positive, except underlined values, which represent negative correlations.

CVL 2003 (left), 2006 (right)

	<u>Ca</u>	<u>Cu</u>	<u>Fe</u>	<u>K</u>	<u>Mg</u>	<u>Mn</u>	<u>P</u>	<u>S</u>	<u>Zn</u>
Ca	*	0.0625	0.1983	<u>0.004</u>	0.0432	0.0823	0.1375	0.0428	0.2185
Cu	<u>0.0079</u>	*	0.2336	0.122	0.136	0.0921	0.0804	0.0315	0.2797
Fe	0.047	0.0638	*	0.022	0.1009	0.2272	0.1205	0.0124	0.3319
K	<u>0.0464</u>	0.0078	0.0179	*	0.0716	0.0194	0.1504	<u>0.00005</u>	0.0707
Mg	0.1195	0.0639	0.0758	0.1644	*	0.0765	0.3956	0.0082	0.3677
Mn	<u>0.0027</u>	0.1277	0.0603	0.022	0.104	*	0.1563	0.0214	0.1012
P	0.1894	0.0428	0.1047	0.2482	0.4281	0.0317	*	0.0118	0.3663
S	0.0094	0.0039	<u>0.0221</u>	0.0561	0.0561	0.0002	0.1056	*	0.0018
Zn	0.1056	0.1678	0.3666	0.0127	0.2334	0.1996	0.2184	<u>0.0041</u>	*

CVL hulls (right), CL 2003 (left)

	<u>Ca</u>	<u>Cu</u>	<u>Fe</u>	<u>K</u>	<u>Mg</u>	<u>Mn</u>	<u>P</u>	<u>S</u>	<u>Zn</u>
Ca	*	<u>0.0176</u>	0.0104	<u>0.0659</u>	0.2041	0.0133	0.1024	0.015	0.0129
Cu	0.2126	*	0.0515	0.0295	0.0057	0.017	0.096	0.0703	0.1234
Fe	0.303	0.2556	*	<u>0.0248</u>	0.0023	0.0339	0.0073	0.007	0.1
K	0.0253	0.319	0.0696	*	<u>0.0015</u>	<u>0.0003</u>	0.0548	0.0666	0.0005
Mg	0.0311	0.2444	0.0178	0.3375	*	0.0953	0.1548	0.0446	0.0778
Mn	0.0646	0.2058	0.2266	0.0039	0.0065	*	0.0142	0.0029	0.2192
P	0.2707	0.2888	0.224	0.2949	0.293	0.00005	*	0.2439	0.0289
S	0.0692	0.0126	0.0029	0.0303	0.0049	<u>0.0005</u>	0.0228	*	0.0038
Zn	0.2199	0.4379	0.3213	0.2584	0.135	0.1556	0.3001	0.0185	*

CL 2007 (right), 2005 (left)

	<u>Ca</u>	<u>Cu</u>	<u>Fe</u>	<u>K</u>	<u>Mg</u>	<u>Mn</u>	<u>P</u>	<u>S</u>	<u>Zn</u>
Ca	*	0.132	0.0459	<u>0.2029</u>	0.0551	0.2634	0.0867	0.2548	0.1456
Cu	0.3331	*	0.2146	0.0066	0.2366	0.1201	0.1318	0.0409	0.4242
Fe	0.0573	0.289	*	<u>0.0085</u>	0.108	0.1162	0.0472	0.0137	0.3921
K	<u>0.0172</u>	0.0509	0.0351	*	0.1267	0.1389	0.1669	<u>0.0718</u>	<u>0.0221</u>
Mg	0.1539	0.3958	0.1478	0.163	*	0.0014	0.4425	0.0071	0.1639
Mn	0.2117	0.2061	0.1481	<u>0.0259</u>	0.102	*	0.03	0.0736	0.264
P	0.3111	0.2504	0.0525	0.1026	0.3983	0.0669	*	0.0059	0.1005
S	0.1303	0.0644	0.0061	0.0772	0.0043	0.0836	0.0051	*	0.0605
Zn	0.1655	0.4202	0.3804	0.0015	0.2454	0.5217	0.1285	0.0542	*